From the Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

TUMOR-INFILTRATING LYMPHOCYTES AND THE PD-L1/PD-1 SIGNALING AXIS IN BREAST CANCER: BIOLOGY AND CLINICAL IMPLICATIONS

Ioannis Zerdes

Karolinska Institutet

Stockholm 2020

Cover image: Tissue microarray core derived from breast cancer tissue, stained with multiplex fluorescent immunohistochemistry

Published by Karolinska Institutet.
Printed by E-Print AB 2020

ISBN 978-91-7831-780-6

Tumor-infiltrating lymphocytes and the PD-L1/PD-1 signaling axis in breast cancer: biology and clinical implications

THESIS FOR DOCTORAL DEGREE (Ph.D.)

To be defended in public on April 24, 2020 at 09.30 a.m.

A4:04 Jan Lindsten, New Karolinska University Hospital, Stockholm

By

Ioannis Zerdes, M.D.

Principal Supervisor:
Associate Professor Theodoros Foukakis
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisors:
Professor Jonas Bergh
Karolinska Institutet
Department of Oncology-Pathology

Adjunct Professor Georgios Rassidakis
Karolinska Institutet
Department of Oncology-Pathology

Associate Professor Charlotte Rolny
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Assistant Professor Michail Ignatiadis
Medical Oncology Department, Institut Jules Bordet & Université Libre de Bruxelles, Brussels, Belgium

Examination Board:
Associate Professor Margareta Wilhelm
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Associate Professor Patrick Micke
Uppsala University
Department of Immunology, Genetics and Pathology

Associate Professor Simon Ekman
Karolinska Institutet
Department of Oncology-Pathology

To all those who believed in me

ABSTRACT

Cancer immunotherapy and especially immune checkpoint blockade therapy has revolutionized cancer treatment in many tumor types including breast cancer (BC). The expression and specific role of Programmed Death-1 (PD-1)/Programmed Death-Ligand 1 (PD-L1) signaling axis, immune-related gene signatures and immune cell subpopulations needs to be fully elucidated while the identification of prognostic and predictive biomarkers in BC is of utmost importance.

The overall aim of the thesis was to investigate the expression, prognostic/predictive implications and regulatory mechanisms of the PD-1/PD-L1 checkpoints and immune infiltrate in early and advanced BC.

In paper I, a comprehensive analysis of PD-L1 expression patterns and its prognostic implications was performed in early BC. PD-L1 protein expression was mostly expressed in immune cells and was most abundant in the TNBC subtype. PD-L1 expression in tumor cells was a poor prognostic factor whereas PD-L1 expression in immune cells was correlated with improved survival outcomes in TNBC, as revealed in a trial-level meta-analysis including 38 studies. PD-L1 gene expression was associated with improved prognosis in the entire population as revealed in a pooled transcriptomic analysis of 39 publicly available datasets. PD-L1 expression can therefore represent a promising clinically relevant biomarker of good prognosis which can also select appropriate candidates for immunotherapy.

In paper II, PD-L1 expression was evaluated both at the protein and mRNA level in the same retrospective early BC patient cohort. Both protein (stained with the antibody clone SP263) and gene expression predicted improved outcome in early BC and also correlated with enhanced T-cell infiltration (CD3+ IHC and in silico estimation of CD8+/CD4+ T-cells). Of note, PD-L1 mRNA expression can add significant prognostic value to the prospectively validated 21- and 70-gene gene signatures in ER+/HER2- disease. Upon validation, this finding might improve prognostication capacity of the current gene signatures.

In paper III, we investigated the role of STAT3 as a regulator of PD-L1 expression and immune response in BC *in vitro*, *in vivo* and in BC patient samples. A positive correlation between STAT3 and PD-L1 was observed at the protein and gene expression level while a transcriptional STAT3-mediated regulation of PD-L1 was demonstrated in BC cells. Of note, STAT3 modulated antitumor immune response mainly through macrophage phenotype shift and accumulation of NK cells rather than via cytotoxic T-cell infiltration in a murine BC model. Furthermore, pro-tumoral macrophages were correlated with PD-L1 expression in BC patient tumors, thus providing insights on the tumor-immune cell interactions and potential clinical implications.

In paper IV, a multi-level study of the prognostic capacity of PD-1 expression in early BC was performed including: a) a study cohort with IHC and GEP data, b) systematic review and trial-

level IHC-based meta-analysis of 15 studies and c) pooled analysis of publicly available transcriptomic datasets). PD-1 protein and gene expression were correlated with improved OS in the entire population of the study cohort. In the pooled analyses PD-1 protein and gene expressions were correlated with better survival outcomes in TNBC/basal-like patients and therefore is a promising biomarker which merits further validation.

In paper V the role of relevant gene signatures as predictors of response to chemotherapy was investigated in patients (n=109) with advanced BC participating in the translational sub-study of the phase III TEX trial. Fine-needle aspiration biopsies were used for gene expression profiling and for TILs enumeration. Immune-related gene signatures predicted better response to chemotherapy in ER+ and luminal BC patients which was further confirmed through an independent gene set enrichment and other *in silico analyses*. The lymphocytic abundance was low and predicted no effect to chemotherapy. These results may pave the way for the development of immune-based drivers of chemosensitivity in the least immunogenic luminal tumors.

LIST OF SCIENTIFIC PAPERS

I. Matikas A[§], **Zerdes I**, Lövrot J, Richard F, Sotiriou C, Bergh J, Valachis A[#], Foukakis T[#]**.**
Prognostic implications of PD-L1 expression in breast cancer: systematic review and meta-analysis of immunohistochemistry and pooled analysis of transcriptomic data
Clinical Cancer Research 2019 Sep 15;25(18):5717-5726

II. **Zerdes I[§]**, Sifakis EG, Matikas A, Chrétien S, Tobin NP, Hartman J, Rassidakis GZ, Bergh J, Foukakis T.
Programmed Death Ligand-1 gene expression is a prognostic marker in early breast cancer and provides additional prognostic value to 21-gene and 70-gene signatures in estrogen receptor-positive disease.
Molecular Oncology 2020 Mar 1 (version of record online: 20 March 2020)

III. **Zerdes I[§]**, Wallerius M, Sifakis EG, Wallmann T, Betts S, Bartish M, Tsesmetzis N., Tobin NP, Coucoravas C., Bergh J., Rassidakis GZ., Rolny C., Foukakis T.
STAT3 activity promotes Programmed-Death Ligand 1 expression and suppresses immune responses in breast cancer.
Cancers (Basel) 2019 Oct 1;11(10). pii: E1479

IV. **Zerdes I*[§],** Matikas A*, Lövrot J, Sifakis EG, Richard F, Sotiriou C, Rassidakis GZ, Bergh J, Valachis A, Foukakis T.
PD-1 protein and gene expression as prognostic factors in early breast cancer.
Manuscript

V. Foukakis T [§], Lövrot J, Matikas A, **Zerdes I**, Lorent J, Tobin N, Suzuki C, Brage SE, Carlsson L, Einbeigi Z, Linderholm B, Loman N, Malmberg M, Fernö M, Skoog L, Bergh J, Hatschek T.
Immune gene expression and response to chemotherapy in advanced breast cancer.
British Journal of Cancer 2018 Feb 20;118(4):480-488

*Denotes equal first-author contribution

[#] Denotes equal last-author contribution

[§] Denotes corresponding author

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. **Zerdes I**, Matikas A, Bergh J, Rassidakis GZ, Foukakis T[§].
Genetic, transcriptional and post-translational regulation of the programmed death protein ligand 1 in cancer: biology and clinical correlations.
Oncogene 2018 Aug;37(34):4639-4661

II. Chrétien S, **Zerdes I**., Bergh J, Matikas A, Foukakis T[§].
Beyond PD-1/PD-L1 inhibition: what the future holds for breast cancer immunotherapy.
Cancers (Basel) 2019 May 5;11(5). pii: E628

III. González-Rodríguez P, Engskog-Vlachos P, Zhang H, Murgorci AN, **Zerdes I**, Joseph B[§].
SETD2 mutation in Renal Clear Cell Carcinoma suppress autophagy via regulation of ATG12.
Cell Death & Disease 2020 Jan 27;11(1):69

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIMS	Absolute Intrinsic Molecular Subtyping
AP-1	Activator Protein 1
APC	Antigen-Presenting Cells
BRISQ	Biospecimen Reporting for Improved Study Quality
BSA	Bovine Serum Albumin
BC	Breast Cancer
CIBERSORT	Cell type identification by estimating relative subsets of RNA transcripts
CISH	Chromogenic In Situ Hybridization
CPS	Combined Positive Score
C-index	Concordance Index
CI	Confidence Interval
CTLs	Cytotoxic T-Lymphocytes
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
DC	Dendritic Cells
DFS	Disease-Free Survival
DMFI	Distant Metastasis-Free Interval
DMFS	Distant Metastasis-Free Survival
DRFI	Distant Recurrence-Free Interval
DCIS	Ductal Carcinoma In Situ
ER	Estrogen Receptor
EFS	Event-Free Survival
ERK	Extracellular signal-Regulated Kinase
FDR	False Discovery Rate
FNAB	Fine-Needle Aspiration Biopsy
FISH	Fluorescence In Situ Hybridization
FEC	Fluorouracil, Epirubicin and Cyclophosphamide
FDA	Food and Drug Administration
FoxO1	Forkhead box O1

FoxP3	Forkhead box P3
FFPE	Formalin-fixed paraffin-embedded
GEO	Gene Expression Omnibus
GEP	Gene Expression Profiling
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GS	Gene Signatures
GGI	Genomic Grade Index
HR	Hazard Ratios
H&E	Hematoxylin-Eosin
HR	Hormone receptors
HER2	Human Epidermal Growth Factor Receptor 2
HIF-1	Hypoxia Inducible Factor 1
ICB	Immune Checkopoint Blockade
IHC	Immunohistochemistry
ITT	Intention-To-Treat
IFN-γ	Interferon-gamma
IL-	Interleukin-
IQR	Interquartile Range
JAK	Janus Kinase
KM	Kaplan–Meier
LR	Likelihood Ratio
LN	Lymph Node
LPBC	Lymphocyte-Predominant Breast Cancer
MHC	Major Histocompatibility Complex
mTOR	mechanistic Target Of Rapamycin
MCP-counter	Microenvironment Cell Populations counter
miRNA	microRNAs
METABRIC	Molecular Taxonomy and Breast Cancer International Consortium
MDSCs	Myeloid-Derived Suppressor Cells
NK cells	Natural-Killer cells

NACT	Neoadjuvant Chemotherapy
NAT	Neoadjuvant Treatment
NFATc1	Nuclear Factor of Activated T-cells, cytoplasmic 1
NF-κB	Nuclear Factor-kappa Beta
ORR	Objective Response Rate
OR	Odds Ratio
OS	Overall Survival
pCR	pathologic Complete Response
PBS	Phosphate-Buffered Saline
PIK3CA	Phospatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha
pSTAT3-GS	phosphorylated STAT3-associated Gene Signature
PVDF	Polyinylidene Difluoride
PCR	Polymerase Chain Reaction
PAM50	Prediction Analysis of Microaaray 50
PR	Progesterone Receptor
PD-1	Programmed Death 1
PD-L1	Programmed Death Ligand-1
PFI	Progression-Free Interval
PFS	Progression-Free Survival
PH	Proportional Hazards
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
ROC	Receiver Operating Characteristic
RS	Recurrence Score
RFS	Relapse-Free Survival
REMARK	REporting recommendations for tumor MARker prognostic studies
RECIST	Response Evaluation Criteria In Solid Tumors
ROR	Risk of Recurrence
RNA-seq	RNA-sequencing
RMA	Robust Multichip Averaging
STAT3	Signal activation of Transcription 3
sTILs	Stromal Tumor-Infiltrating Lymphocytes

TCR	T-cell receptor
Th1	T-helper 1
T-regs	T-regulatory cells
TLS	Tertiary-Lymphoid centers
TCGA	The Cancer Genome Atlas
TTF	Time to Treatment Failure
TMA	Tissue Microarrays
T-DM1	Trastuzumab emtansine
TNBC	Triple-Negative Breast Cancer
TBS	Tris-Buffered Saline
TIMER	Tumor Immune Estimation Rescource
TME	Tumor MicroEnvironment
TMB	Tumor Mutational Burden
TAM	Tumor-Associated Macrophages
TIME	Tumor-Immune MicroEnvironment
TILs	Tumor-Infiltrating Lymphocytes
WHO	Word Health Organization

1 INTRODUCTION

Breast cancer (BC) is the most common malignancy and the leading cause of cancer death in women worldwide (1). Owing to advances in treatment and early detection by screening (2), death rates in BC have decreased and 5-year survival presently exceeds 75% in most western countries (3, 4). In the metastatic setting, however the disease is not curable and treatment has a palliative intention. Despite an extended arsenal of different cytotoxic, endocrine and targeted therapies the long-term prognosis is poor with a median survival of less than two years in the population level (5).

Breast cancer is a heterogeneous disease both clinically and biologically. According to the immunohistochemical expression of hormone receptors (HR) (estrogen receptor, ER; progesterone receptor, PR) and human epidermal growth factor receptor 2 (HER2), there are three major different BC subtypes:

i. ER-positive, HER2-negative (ER+/HER2-), which represents the most common subtype (approx. 70%). Although it is correlated with the best prognosis, a steady rate of recurrence has been described even 20 years after endocrine threrapy (6).
ii. HER2-positive (HER2+) (approx. 20% of the cases); it was initially associated with adverse clinical outcome (7) but the development of anti-HER2 targeted agents has revolutionized the management of these patients, thus prolonging survival rates.
iii. Triple-Negative BC (TNBC) where none of the aforementioned markers are expressed (approx. 10-20%) . This subtype is the most aggressive one, associatted with the worst survival outcomes (8).

Moreover, apart from the clinical subtypes which can indicate the underlying molecular BC heterogeneity, genomic studies have also revealed four distinct biological “intrinsic” subtypes. These include i) luminal A, ii) luminal B, iii) HER2-enriched and iv) basal-like (9).

1.1 Adjuvant and neoadjuvant treatment in breast cancer

The use of adjuvant polychemotherapy is associated with benefits in relapse-free survival (RFS) and overall survival (OS) in patients with early BC, regardless of clinicopathological factors (10). Recently the concept of dose-dense adjuvant chemotherapy administration has resulted into moderately reduced 10-year risk of recurrence and death from BC as compared to standard-schedule chemotherapy administration (11). Furthermore, in patients with

HR+/HER2- and HER2+ non-metastatic BC, endocrine therapy and HER2-targeted treatment should be offered, respectively (12).

On the other hand, the use neoadjuvant treatment (NAT) leads to increased rates of breast-conserving surgery and favorable long-term survival outcomes in patients achieving pathologic complete response (pCR) (13). The efficacy of neoadjuvant chemotherapy is shown to be equivalent to adjuvant chemotherapy (14), thus enabling the development of clinical trials and translational research platforms (i.e. investigation of tumor biology and heterogeneity).

However, there are still patients who will experience disease relapse and others who will be exposed to unnecessary toxicities due to overtreatment. Therefore, there is a need for identification of reliable biomarkers which can stratify patients at risk and also guide treatment decisions.

1.2 Prognostic and predictive biomarkers in breast cancer

A biomarker/factor is prognostic when it can inform about the clinical outcome irrespective of any provided treatment. On the other hand, a predictive biomarker informs about the potential benefit that patients could gain from a specific therapeutic intervention (15). In order a biomarker to be implemented in clinical routine, it should prove its analytical validity (which reflects the technical issues of a test including accuracy and reproducibility), clinical validity (which reflects the capacity of a factor to distinguish subgroups with different biological/clinical outcomes) and clinical utility (which reflects usefulness of a factor for direct patient care and its ability to impact outcome when used), principles ensuring that a factor can be precise, reproducible, easily interpretable (16, 17). In BC, known prognostic factors include: age, race, pathologic factors (such as tumor stage, tumor size, lymph node involvement, presence of metastatic disease, tumor morphology, histologic grade, perivascular lymphovascular invasion) as well as tissue markers (i.e. ER and HER2 status represent prognostic factors and in addition they can predict benefit of treatment with hormonal and anti-HER2 therapy, respectively) and proliferation markers (e.g Ki-67) (18, 19). Furthermore, the optimization and validation of specific gene expression profiling (GEP) patterns has led to the development of relevant biomarkers (intrinsic subtypes and gene signatures), as discussed hereunder.

1.3 Gene expression signatures as prognostic & predictive factors in early breast cancer

The rapid advances in technology have enabled the introduction and wide use of gene expression profiling as a useful tool in clinical medicine (20, 21). Especially in BC, gene expression signatures have been developed in order to predict the risk of recurrence but also importantly to distinguish patients who will benefit from chemotherapy de-escalation strategies. Several gene signatures have been developed namely: 21-gene (Recurrence score-RS, commercially Oncotype DX), 70-gene signature (commercially, Mammaprint), EndoPredict, Prediction Analysis of Microaaray 50 (PAM50) (Risk-of-Recurrence, ROR; commercially, Prosigna) and Genomic Grade Index (GGI) and their prognostic capacity has been compared in previous studies (22). Only two of these gene signatures, the 21-gene and 70-gene signature, have demonstrated their clinical utility through validation in the prospective randomized clinical trials TAILORx (23) and MINDACT (24), respectively. High RS is associated with high risk of recurrence and also predicts benefit from adjuvant chemotherapy in ER-positive/HER2- BC patients (25). By contrast, ER+, node-negative BC patients with low and intermediate RS (for the latter should be also >50 years old) can safely forego chemotherapy (26). In the MINDACT trial, patients with high clinical risk and low genomic risk according to the 70-gene signature had an excellent 5-year distant metastasis-free survival (DMFS) rate (94.7%, 95% CI 92.5 – 96.2) without receiving chemotherapy, although a trend in favor of chemotherapy was noted in this group (24). However, the use of these and the other gene signatures needs to be further refined and some questions remain to be addressed, since their benefit has been shown mostly node-negative patients while the data are limited for the node-positive disease (27).

1.4 Tumor immunology and immunoediting

Cancer development and progression has long been known to raise a anti-tumor immune response through which cancer cells can be eradicated by the immune system, unless they manage to escape immune recognition or create an immunosuppressive environment (28). The concept of immune surveillance describes the complex interactions between immune-host and tumor cells (Figure 1) and comprised three stages: elimination, equilibrium and evasion. Therefore, the ability of tumors to escape from immune destruction has been characterized as a hallmark of cancer (28, 29).

The generation of the complex cyclic process of cancer-immunity interaction leading to the effective anti-tumor immune surveillance (30, 31) includes the following steps: 1. release of

cancer cell antigens (neoantigens) as derived by immunogenic/tolerogenic cancer cell death and processing by antigen-presenting cells (3), 2. presentation of neoantigens to T-cells by APCs on the major histocompatibility complex I (MHC I) and II (MHC II), 3. priming and activation of T-cells against cancer antigens, 4. trafficking and 5. infiltration of effector/cytotoxic T-cells to tumor bed, 6. recognition of cancer cells (via T-cell receptors, MHC and other co-stimulatory molecules) and 7. killing of cancer cells.

The anti-tumor immune response in BC can be different according to the disease subtype. TNBC subtype is considered to be highly heterogeneous and more immunogenic as compared to luminal subtype (32, 33). This trait can also be attributed to its higher mutational load despite that conflicting data have been reported regarding the association between mutational load and host immune response. On the one hand, higher genomic heterogeneity can generate neoantigens and therefore a stronger anti-tumor immune response whereas a decreased immune infiltrate can impair anti-tumor immune surveillance and can trigger intra-tumoral clonal heterogeneity (34).

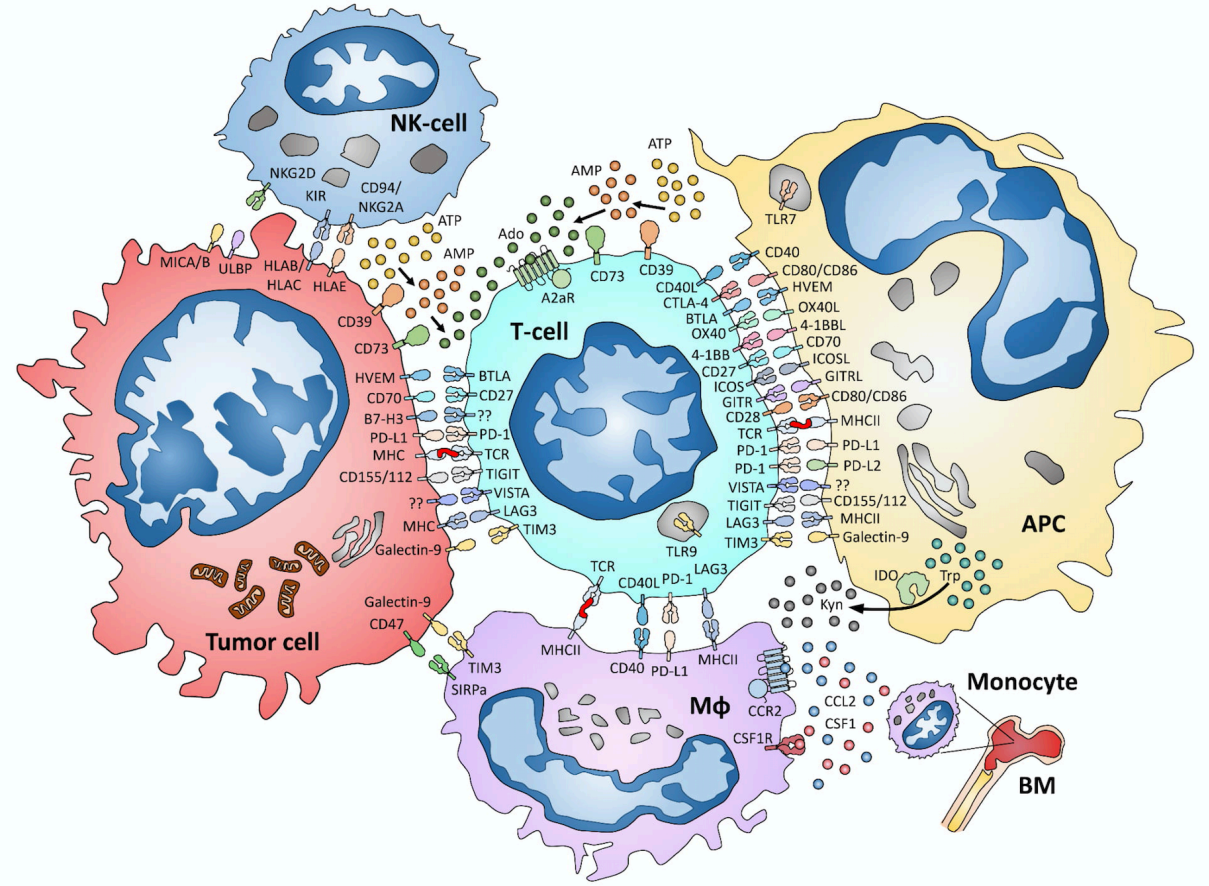

Figure 1. Complex tumor-immune cell interactions in the tumor microenvironment (TME) *(Chrétien et al., Cancers 2019)*

1.5 Tumor infiltrating lymphocytes (TILs) and host immune response as prognostic and predictive biomarkers in early and metastatic breast cancer

1.5.1 Prognostic value

Breast cancer has been traditionally considered as a non-immunogenic or "immune-cold" tumor mainly due to its lower mutational load compared to other tumor types such as melanoma or non-small cell lung carcinoma (35, 36). However, infiltration of breast tumors by TILs is common especially among the TNBC and HER2+ subtypes (37, 38), which is generally reflecting an immunologic response against the tumor.

The presence of lymphocytic infiltrate has been extensively explored as a prognostic biomarker. In early stage TNBC subtype, stromal TILs (sTILs) after adjuvant chemotherapy were independent prognostic factors of improved disease-free survival (DFS) and OS, as revealed in a recent pooled individual patient data analysis (n= 2148) from nine clinical trials (39). Similar associations have been previously reported in prospective-retrospective analyses of clinical trials in TNBC (i.e. BIG 02-98, FinHER, ECOG 2197 and ECOG 1199) (40-42), further supporting the higher immunogenicity/lymphocytic infiltration and its favorable impact on improved survival outcomes. In another large pooled analysis including a total of 3771 patients from six randomized trials from the German Breast Cancer Group, both TNBC and HER2+ subtypes, high levels of TILs have been correlated to improved DFS (43). In contrast, existing data for TILs in ER+ BC (the lowest levels among subtypes) do not support their use as prognostic biomarkers in these tumors (37, 44).

Of note, in a recent paper including a pooled analysis of four cohorts of early TNBC patients (n=476) who were not treated with adjuvant chemotherapy, higher stromal TILs levels were independently correlated with increased DFS and OS. Excellent prognosis has also been demonstrated in patients with pathological stage I and high sTILs, and might represent a target group for chemotherapy de-escalation strategies (45).

The aforementioned observations have led to the characterization of the lymphocyte-predominant breast cancer (LPBC) subtype, defined as ≥50% of the tumor area occupied by TILs as evaluated in H&E stained tumor sections (46) (see also below *TILs evaluation methodology and future perspectives*) which will also be also incorporated in the updated Word Health Organization (WHO) BC classification (47).

1.5.2 Predictive value

1.5.2.1 Response to chemotherapy (neoadjuvant and adjuvant)

Several studies have highlighted the role of TILs as biomarkers for predicting benefit from both adjuvant and neoadjuvant chemotherapy (43, 48).

The phase III randomized GeparSixto trial including TNBC and HER2+ patients who received NAT the combination of anthracyclines-taxanes and trastuzumab +/- carboplatin reported that LPBC patients presented with higher pCR rates when receiving carboplatin, further suggesting that TILs can predict benefit and sensitivity to a specific chemotherapy regimen (48). However, the presence of high lymphocytic infiltration as described in a later study by Denkert et al. can predict response to NAT and leads to increased pCR rates in all subtypes (43).

In the adjuvant setting, the effect of stromal TILs was favorable regardless of the chemotherapy regimen used (anthracycline-based chemotherapy with or without taxanes) in a large TNBC pooled analysis (39). Interestingly, in the secondary analysis BIG 02-98 trial, HER2+ breast patients with high stromal TILs values were significantly associated with benefit from anthracycline-only regimen as compared to anthracycline-docetaxel combination; significant interaction for both DFS and OS was noted (40).

1.5.2.2 Response to anti-HER2 therapies (adjuvant and neoadjuvant)

In the HER2+ subtype, controversial data have been reporting on the predictive role of TILs to adjuvant trastuzumab treatment. In the FinHER study, TILs were significantly associated with survival benefit from trastuzumab (41). In contrast, the N9831 study (n=945) demonstrated an association of high sTILs with improved relapse-free survival only in the chemotherapy arm and not in the trastuzumab (49) while similar results of a non-significant interaction between high sTILs and trastuzumab benefit were demonstrated in the NSABP B-31 study (50).

Regarding treatment duration and immune response, it has been reported in the ShortHER trial that patients with low TILs had a greater treatment benefit from 1-year as compared to 9-weeks trastuzumab (+chemotherapy). On the other hand, patients with high TILs had an improved DFS regardless of trastuzumab duration (51).

In the neoadjuvant setting, TILs have been investigated as pCR predictors in prospective-retrospective analyses of many trials using anti-HER2 therapies. Of note, it was in a meta-analysis including 1,256 patients treated with chemotherapy and trastuzumab, lapatinib (or combination) in five randomized phase 3 trials (CherLOB, NeoALLTO, GeparQuattro, GeparQuinto and GeparSixto), that baseline sTILs levels were associated with higher pCR rates irrespective of the anti-HER2 or chemotherapy agents used (48, 52-55).

1.6 Temporal expression of TILs and prognostic implications in breast cancer

1.6.1 Tumor-infiltrating lymphocytes in the metastatic versus primary breast tumors

The role of TILs in BC metastatic setting has not been fully elucidated since data are scarce. Although consensus recommendations for the evaluation of TILs include metastatic tissues (56), their evaluation has not always proven to be successful (see also paper V) (57) and remains challenging. In the CLEOPATRA randomized phase 3 trial, including 678 HER2+ metastatic BC patients with evaluable tumor samples, higher levels of stromal TILs were significantly associated with prolonged OS, regardless of treatment group (docetaxel and trastuzumab combined with either pertuzumab or placebo) (58). In contrast, in another randomized phase 3 study (The Canadian Cancer Trials Group MA.31 trial) of metastatic HER2-positive patients receiving trastuzumab or lapatinib in combination with taxane, no significant prognostic or predictive effect was observed in the high-TILs group (59). Moreover, a few previous reports have demonstrated that the number of TILs was lower in metastases compared to the primary tumor, especially in the TNBC and HER2+ subtypes (60-63). Metastatic BC patients with higher TILs had improved survival as compared to those with lower TILs expression levels (62). Among specific immune cell subpopulations, CD4+ and CD8+ T-cells also significantly decreased in the metastatic lesions (60, 61), while some studies have also investigated the differential expression of T-regulatory cells (T-regs) (60, 62). Of note, macrophage expression markers evaluated by gene expression profiling remained highly expressed in the metastatic sites (63), while CD68+ IHC expression in BC brain metastases was independently associated with improved survival (64).

1.6.2 Pre-treatment and post-treatment levels and association with pCR

The dynamic expression of immune lymphocytic infiltration has been evaluated in pre- and post-treatment samples of BC patients treated with neoadjuvant chemotherapy (NACT). In a large series of 716 patients with paired pre- and post-treatment samples, TIL levels significantly decreased after completion of chemotherapy (pre-NACT mean TILs= 24.1% versus post-NACT mean TILs=13.0%) and this decrease was associated with high pCR rates (65). In examined patient samples from the SWOG S0800 trial, TILs levels significantly decreased in the pCR cases after treatment while remained similar with the pre-treatment levels in patients the residual disease; no association with survival outcomes was observed (66). Of note, in HR+/HER2- patients, sTILs and CD8+ T-cells were significantly decreased in post-treatment samples while gene expression profiling revealed that immune gene-based cell subpopulations

corresponding T-regs was also downregulated (67). Regarding lymphocytic subpopulations, significantly decreased in CD4+, CD20+ and CD68+ levels were observed in post-treatment samples. High decrease of CD4+ and CD20+ and CD3+ cells were associated with pCR and also with better survival outcomes (especially for the CD3+) (68).

1.6.3 Residual disease after neoadjuvant chemotherapy

Given that non-pCR BC patients treated with neoadjuvant therapy are associated with worse prognosis (13), the evaluation of TILs infiltration in the residual disease and its effect on long-term outcomes represent an emerging research topic of high interest.

In TNBC, a recent study derived from four patient cohorts (n=375) has shown that higher residual disease TILs were significantly correlated to improved RFS and OS (69) and similar results have been also derived from another retrospective multicenter study (n=278) (70). When different immune cell populations were evaluated in the residual tumors of TNBC patients, high CD8+ TIL levels and a high CD8/FOXP3 ratio significantly correlated with prolongs recurrence-free and breast cancer-specific survival (71).

Apart from the evaluation of stromal TILs in the residual disease, enrichment of immune-related gene signatures correlated with favorable prognosis mainly in the TNBC subtype (72, 73). These findings indicate TILs as a new surrogate endpoint, underscores the need for evaluating on-treatment immune infiltration and can improve prognostication in non-pCR TNBC patients after neoadjuvant chemotherapy (74). Indeed, the reported results of the CREATE-X randomized phase 3 trial in patients who did not achieve pCR, indicated that administration of adjuvant capecitabine improved both 5-year DFS (74.1% vs 67.6%; HR: 0.70, 95% CI 0.53–0.92, p=0.01) and OS (89.2% vs 83.6%; HR: 0.59, 95% CI 0.39–0.90, p=0.01) as compared to the control group, and especially in the TNBC subgroup (75).

In contrast, in HER2+ BC treated with neoadjuvant chemotherapy (+/- trastuzumab), high TIL levels in with residual disease were significantly independently associated with worse disease-free survival and higher residual tumor burden (76), while an increase of FOXP3+ TILs indicative of an immunosuppressive phenotype has been described in HER2+ residual disease (77).

The predictive capacity of TILs has been also evaluated in combination with other indicators of residual disease such as residual cancer burden (RCB), which represents a combined continuous index of primary tumor (size and cellularity) and nodal metastases (number and size) measurements (78). Of note, Asano and colleagues have shown that combination of RCB with high TILs was a significant independent marker of reduced risk of recurrence in all BC

subtypes (79) while in another study it was demonstrated that TILs can also add prognostic information to RCB (69, 78).

1.7 Immune cell subpopulations and prognostic correlations in breast cancer

Given that all mononuclear cells are included in the scoring of TILs in BC tissue stained with hematoxylin-eosin (H&E) (46, 56), the role of the specific immune cell subpopulations needs to be better clarified. TILs include components of both innate immunity e.g macrophages, natural-killer (NK) cells, dendritic cells (DC) and myeloid-derived suppressor cells (MDSCs) and also adaptive immunity e.g. CD8+ and CD4+ T-cells, T-regs. Each of these major subpopulations within tumor immune microenvironment (80) can influence patient outcomes (81). It has been described earlier that increased inflammatory/immune infiltrate was correlated with improved survival outcomes in more than half of the identified reports (82). Specifically:

Cytotoxic T Lymphocytes (CTLs): The prognostic role of CD8+ cytotoxic tumor-infiltrating lymphocytes has been demonstrated in two large retrospective primary breast cancer studies (n_1=8,978 and n_2=1,334) (83, 84). The presence of CD8+ TILs was significantly independently correlated with improved survival outcomes in both cohorts. Moreover, this effect was seen in the ER-negative/basal-like and HER2-enriched tumors but not in the luminal subtype.

T-regulatory cells: This type of T-cells is immunohistochemically characterized by the nuclear expression of Forkhead box P3 (FoxP3) and is generally considered as immunosuppressive and linked to tumor progression (85). In a large retrospective analysis of three studies (n=5239), FoXP3+ cells were not correlated with breast cancer-specific survival (83) whereas in another large meta-analysis (n=8,666), patients with high FoXP3+ TILs were associated with worse overall survival (86). Of note, the CD8/FoxP3 ratio represents an immune activation parameter and a higher ratio has been linked to better survival in ER-negative BC patients (87).

CD4+ T-cells subsets: Apart from the T-regs, CD4+ T-helper subsets include T-helper 1 (Th1) Th2, Th17 and follicular helper T-cells (Tfh) (88). Th1 and Tfh have been associated with favorable DFS and OS in BC patients (89). In contrast Th2 and Th17 have been mostly correlated to immune escape and poorer prognosis (90).

Myeloid-derived suppressor cells: MDSCs denote a heterogeneous population of myeloid cells (CD33+ and CD11b+) which promote an immunosuppressive microenvironment, tumor progression and metastatic spread. In BC, higher levels of MDSCs (circulating or infiltrating) were correlated to poorer survival and low pCR rates (91-93).

B-cells: Tumor-infiltrating B-cells have been characterized as a cell type linked to functional humoral immune response (94) and there are also present in breast cancer tertiary-lymphoid centers (TLS) (95) . B-cell expression (CD20+ cells) has been correlated with favorable outcome (96) but since it has dual pro- and anti-tumorigenic properties (97), a B-regulatory cell phenotype has been recently recognized to be an adverse prognostic factor combined with T-regs in BC patients (98).

Tumor-associated macrophages (TAMs): This heterogeneous immune cell subpopulation plays a major role in immune evasion, angiogenesis and metastasis and it is roughly divided in two distinct subtypes: the "M1-like" and "M2-like" macrophages which have anti- and pro-tumoral properties respectively (99). Breast cancer patients with high TAM density (mostly of CD68+ and CD163+ macrophages) were associated with worse prognosis as demonstrated in large meta-analysis of sixteen studies (HR=1.50; 95% CI 1.20-1.88 and HR=2.22; 95% CI 1.71-2.89 for OS and DFS, respectively) (100).

1.8 TILs evaluation methodology and future perspectives

TILs in early BC are currently detected under light microscope in H&E stained tissue sections according to the established guidelines by the International TILs Working Group (46), which were adapted for TILs evaluation in Ductal Carcinoma In Situ (DCIS) and in metastatic tumor deposits (56) and also further updated for the TILs scoring in residual disease after NAT (101). According to these recommendations general scoring is performed for lymphocytes and plasma cells, excluding polymorphonuclear lymphocytes. TILs should be scored preferably in whole tissue sections (4-5um of thickness) as compared to biopsies and the average assessment should be done in order the whole tumor area to be covered, instead of focusing on hotspots. The scoring should include only sTILs and should be reported as the percentage (%) of the stromal compartment covered by TILs. TILs in the stroma area should be in direct contact with breast carcinoma cells.

However, no optimal cut-off for TILs abundance has been formally established and needs to be addressed along with the investigation of their clinical utility. Better understanding may lead to integration of TILs as biomarkers in routine clinical care though the development of prognostic risk models or as tools for better patient selection in future immunotherapy clinical trials.

Of note, the latest advances in artificial intelligence and digital pathology (102, 103) could also lead to the optimization and standardization of TILs evaluation in BC, thus increasing

reproducibility and accuracy of scoring (104). In a recent work, automated TILs evaluation (eTILS%) in TNBC using an open source algorithm launched a new method for lymphocytic infiltration assessment (105). High eTILs% score generated using the algorithm previously reported in melanoma (106) was independently associated with improved OS in four TNBC patient datasets (105).

Furthermore, multiplex imaging methods (107, 108) could provide a better insight in the TIME composition and in how the different lymphocytic and macrophage subpopulations and interactions can influence tumor biology, patient survival outcomes.

1.9 The role of immune-related genes and signatures as biomarkers in breast cancer

Moving forward from the morphologic evaluation of TILs to gene expression-based profiling, relevant immune signatures have been investigated as an alternative way of evaluating the immune microenvironment of the tumor and as potential prognostic and predictive biomarkers in BC.

A variety of *in silico* computational approaches and bioinformatic tools have been developed (109) for the deconvolution of tumor immune microenvironment including: i) CIBERSORT (Cell type identification by estimating relative subsets of RNA transcripts) (for the vigorous enumeration of 22 hematopoetic subsets from RNA mixtures; LM22 gene signature matrix) (110) , ii) ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) (111), iii) MCP-counter (Microenvironment Cell Populations counter) (quantification and estimation of the absolute abundance of immune and stromal cell populations based on gene expression) (112), iv) TIMER (Tumor Immune Estimation Rescource; estimation of the abundance of six immune cell types, namely B cells, CD4 T cells, CD8 T cells, neutrophils, macrophages and dendritic cell in TME) (113), v) xCELL (novel gene signature-based method for determination of 64 immune and stromal cell types) (114) and vi) the general tool Gene Set Enrichment Analysis (GSEA)/ Gene Ontology (GO) Enrichment analysis (differential genome-wide expression profiles; can be used for TIME evaluation with a focus on immune pathways) (115). For detailed description of the tools used in the thesis see below (*Patients and methods: Bioinformatic tools for in silico analysis of immune cell subpopulations and tumor microenvironment*).

Additionally, the emerging role of single-cell sequencing approaches could be utilized for the comprehensive characterization immune cell composition in BC (116). These new technologies can be valuable tools for the better understanding of TIME and for the design of novel immunotherapies in (breast) cancer (117, 118).

The advances in gene expression profiling and RNA-sequencing have led to the emergence of immune response-related genes as tools for grasping the complexity of tumor-immune interactions as well as their potential as biomarkers across tumor types (119, 120).

It has been described that immune-related gene signatures can predict increased pCR rates in BC subtypes in the neoadjuvant setting. In the GeparSixto neoadjuvant trial, a 12-gene immune-relevant signature including T-cell, B-cell chemokine and immune checkpoint-related mRNA markers has been derived from 481 breast tumors and differentially correlated to sTILs levels. Of note, all the immune-related genes were significantly associated with increased pCR, while at the single gene level the highest odds ratio was observed for *PD-L1* gene which also represented an independent predictor of pCR (multivariate model included also sTILs). Some genes were also correlated with prediction of carboplatin response (48). Similarly, the favorable effect of enriched immune-related gene signatures as predictive markers to neoadjuvant chemotherapy has been also described by Sota and colleagues who developed an immune-related 23-gene signature which predicted pCR irrespective of the NACT regimens (121). Ignatiadis et al, also reported that high expression levels of T cell-related immune signatures can predict response to neoadjuvant anthracycline +/- taxane -based chemotherapy (122). Similarly, it has been recently described in a secondary analysis of the GeparSepto trial that mRNA-based immune-cell activity (immune "hot/warm" patients) can independently predict neoadjuvant chemotherapy response and long-term prognosis in HER2-negative BC patients (123). Moreover, in the HER2+ patient population, high expression levels of T-cell-related immune signatures were predictive of response to neoadjuvant lapatinib +/- trastuzumab in the NeoALTTO trial (124).

In the adjuvant setting, two large randomized phase III trial have reported the association of immune-related genes to anti-HER2 antibody treatment effect. In the N9831 trial, early BC patients with an enriched expression of a 14-gene immune signature had significant benefit from the addition of adjuvant trastuzumab (125). Furthermore, in an additional translational sub-study of the NSABP-31, patients with high expression of a surrogate gene expression signature of TILs (TILs-related genes) correlated to adjuvant trastuzumab benefit when it is added to doxorubicin-cyclophosphamide + paclitaxel chemotherapeutic combination (126).

Taken together, a variety of evaluation methods for immune cell infiltrate have been comprehensively investigated in a recent study in breast cancer (127). The approaches included sTILs H&E and IHC staining of immune cell markers and manual/digital assessment in whole tissue sections as well as in TMAs. Transcriptomic and methylomic-based approaches were also examined and important differences were observed in the different methods for the evaluation of overall immune infiltration.

1.10 The Programmed Death-1/Ligand-1 (PD-1/PD-L1) signaling axis in cancer

Programmed Death 1 (PD-1, CD279) is a 40kDa cell surface protein (transmembrane type 1) and a member of CD28/CTLA-4 family and immunoglobin superfamily. It is encoded by *PDCD1* gene located on 2q37.3 chromosome (128). PD-1 is mainly expressed on activated T cells but it can also be expressed in other cells such as B cells, macrophages, DC and NK cells (80, 128-131). Programmed Death Ligand 1 (PD-L1, B7-H1, CD274) and PD-L2 (B7-DC, CD273) represent the two ligands of PD-1 and belong to the B7 family of immune-regulatory proteins. PD-L1 is mainly expressed on the surface of tumor cells but it can be also present on various cell types including T cells, B cells, DC, macrophages, mesenchymal stem cells and endothelial cells (132, 133). PD-L1 transmembrane type 1 protein (33kDa) consists of an extracellular immunoglobulin V-like and C-like domains, a hydrophobic transmembrane region, and a 30-amino acid cytoplasmic tail. PD-L2 is almost exclusively expressed on APC. PD-L1 and PD-L2 are encoded by *CD274* and *PDCD1LG2* genes, respectively, located on chromosome 9p.24.1 PD-1/PD-L1 axis is considered as an "immune checkpoint" with crucial impact on autoimmunity (by suppression of cytokine production and T-cell repertoire) , chronic infection and anti-tumor immune response (134). A two signal-activation model is required for T-cell: i) APC and the MHC presented on the surface from T-cell receptor (TCR) (first signal) for antigen recognition and ii) co-stimulatory interaction between CD28 (T-cell surface) and CD80 (B7.1) or CD86 (B7.2) (APC surface) (second signal) (135, 136). Therefore, the engagement of PD-1 with its ligands PD-L1 or PD-L2 preceding the impairment of effective cytotoxic T cell action and proliferation, increased differentiation of T-regs and thus immune cell dysfunction (137) (Figure 2). Blocking the PD-1/PD-L1 signaling axis with monoclonal antibodies can reverse the ineffective anti-tumor immunity and this has proved to be an effective therapy in several cancer types as discussed below in detail (138, 139).

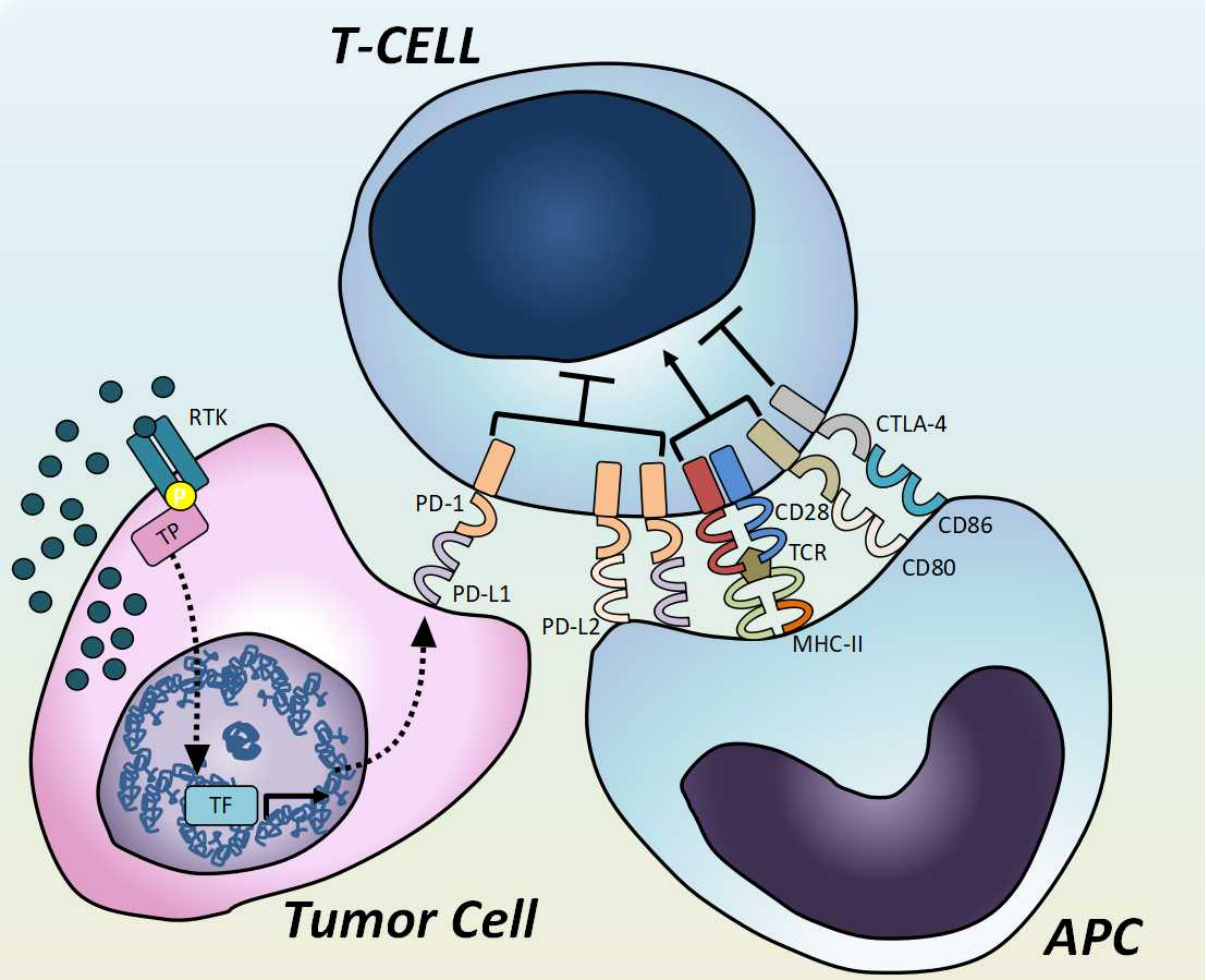

Figure 2. PD-L1/PD-1 signaling axis and interactions among tumors cells, T-cells and antigen-presenting cells

1.11 PD-L1/PD-1 regulation in cancer

Despite the success of immunotherapy in clinical practice, the elucidation of tumor-immune system interplay complexity and underlying mechanisms which govern PD-L1 expression is of high importance. The biology of PD-L1 regulation is complex and includes genetic, transcriptional, signaling pathway, post-transcriptional as well as post-translational regulatory networks (Figure 3) (140). Further understanding of PD-L1 regulation may lead to the introduction of combinational treatments of immunotherapy and targeted therapies, already available for clinical implementation.

The genetic basis of PD-L1 expression has been described and varies among various cancer types. One of the most common copy number alterations is amplification of PD-L1 locus which may lead to elevated PD-L1 protein levels. PD-L1 amplification was most prevalent in

Hodgkin's lymphoma and those patients highly responded to immune checkpoint blockade therapy (141). Therefore, given the challenges on PD-L1 protein evaluation, PD-L1 amplification could serve as alternative for identifying patients gaining the most benefit of immunotherapy. In a large retrospective study including 118,187 solid tumor samples from more than 100 types, PD-L1 amplification was a rare event since it was detected in only 0.7% of the cases (1.9% among breast tumors) and therefore its clinical usefulness remains questionable (142).

A number of oncogenic signaling pathways including Janus Kinase (JAK)/Signal activation of Transcription (STAT), RAS/extracellular signal-regulated kinase (ERK) and/or phosphatidylinositol 3-kinase (PI3K)/AKT/mechanistic Target Of Rapamycin (mTOR) contribute to the regulation of PD-L1 expression and therefore modulate the anti-tumor immune response (143). At the transcriptional level, a lot of factors can regulate PD-L1 expression either directly via binding to the gene promoter or indirectly by regulating other pathways or epigenetic modifiers. Such transcription factors include signal activation of transcription 3 (STAT3), hypoxia inducible factor 1 (HIF-1), p53, Myc, nuclear factor-kappa beta (NF-κB) and activator protein 1 (AP-1). Especially for STAT3, a major oncogene which has a role in several cellular processes (144), it has been recognized to play key role in the regulation of antitumor immunity. Its interplay with various types of immune cells, cytokines, immune checkpoint molecules and other intrinsic pathways can shape a specific (pro- or anti-) inflammatory/tumorigenic microenvironment and lead to tumor escape from immunosurveillance (145).

PD-L1 is also prone to regulation at the post-transcriptional level and post-translational level with several microRNAs (miRNA) ubiquitination and glycosylation processes to influence the regulation of PD-L1 (140). Nevertheless, the exact mechanisms of PD-L1 regulation represent an area of intensive research and could provide the biological rationale for novel therapeutic strategies.

PD-1 expression is also regulated by a variety of factors in cancer. Signal transduction mediated by cytokines (i.e. IL-2. IL-7, IL-15, IL-21) can enhance PD-1 expression on T-cells (146). Moreover, different mechanisms can also mediate an effect on *PDCD1* gene promoter either through the direct impact of transcription factors such as the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (147), NF-kb/p65 and Forkhead box O1 (FoxO1) or through IL-mediated induction of factors which can enhance gene promoter activity (IL-6 – STAT3, IL-12 – STAT4) (130, 143).

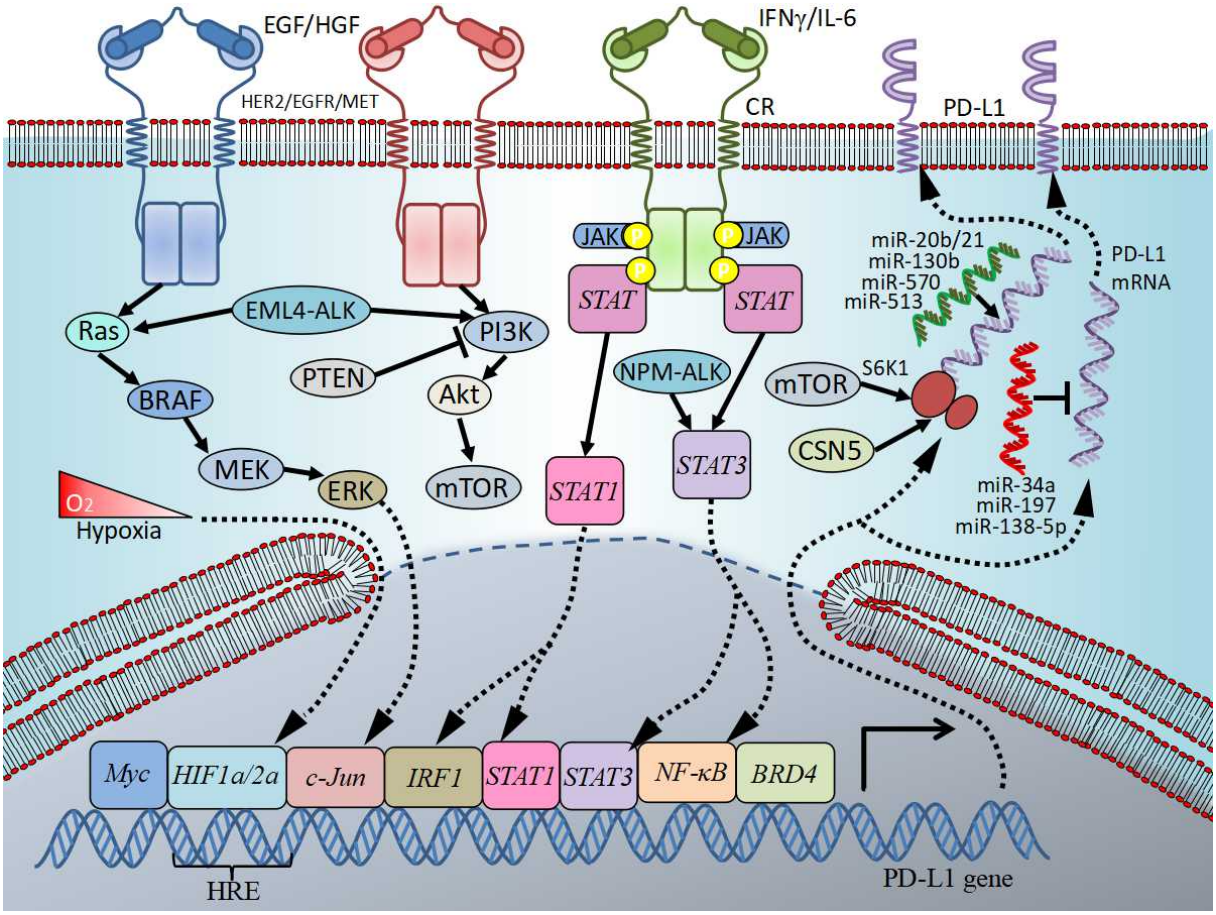

Figure 3. PD-L1 regulation including genetic, transcriptional and post-transcriptional/translational underlying mechanisms *(Zerdes et al., Oncogene 2018)*

1.12 PD-L1 expression in breast cancer: prognostic and predictive implications

The role of the PD-L1 expression as prognostic and predictive biomarker has been extensively investigated in BC. Given that PD-L1 can be expressed in both tumor and immune cells, prognosis and prediction of various treatments mostly depend on the cell of origin, type of tissue (tissue microarrays or whole-tissue sections), BC subtype as well as the disease setting (148). Some previous reports have shown that PD-L1 expression in tumor cells was a negative prognostic factor (149, 150) while some others have demonstrated the correlation of PD-L1 expression with better prognosis in patients with early breast cancer (151, 152). Given that PD-L1 is considered as a co-inhibitory marker leading to impaired anti-tumor immune response this favorable outcome correlation seems indeed to be paradoxical. However, a positive correlation between PD-L1 expression and increased T cell infiltration was reported in a number of studies (153-156), indicating that PD-L1 may represent a surrogate marker/ indicator of immune cell infiltration and activation -which has been related to improved prognosis- rather

than a marker of exhausted T-cells. A possible explanation would involve interferon-gamma (IFN-γ) secreted by TILs which can play a dual role: a) minimize the immune cell infiltration once this is reactive and b) lead to the induction of PD-L1 expression. This procedure is called "adaptive resistance" and indicates a beneficial impact of a pre-existing inflamed immune microenvironment with high -but inhibited- immune cell infiltration (157-159). PD-L1 tumor cell expression has been also correlated with other clinicopathologic characteristics such ER and HER2 negativity, high Ki67, larger tumor size, grade 3 tumors (153).

Hence, many of the previous studies have reported conflicting results and therefore both the role of PD-L1 as a prognostic biomarker in early BC and the overall positivity rates (overall and within subtypes) still remain inconclusive. Furthermore, the meta-analyses that have been previously performed in the topic included a small number of studies and suffer from methodological limitations. A comprehensive analysis on the role of PD-L1 as a prognostic biomarker in early BC is presented in this doctoral thesis (see paper I)(160).

Besides, in order to overcome the analytical and technical difficulties of protein detection, PD-L1 gene expression has been emerged as an alternative approach. Its role as a biomarker needs to be further elucidated, since it has been associated with improved prognosis in early BC patients especially in basal-like subtype (156, 161).

However, the improved prognostication conferred by PD-L1 expression might be attributed to the possible predictive role that PD-L1 expression could have in patients receiving chemotherapy, since we have demonstrated (57, 162) (see also paper V hereunder) that immune-related gene signatures, including PD-L1, are drivers of chemosensitivity. This hypothesis needs to be tested in a prospective or prospective-retrospective randomized manner and in different disease settings. Furthermore, the predictive value of PD-L1 and the concept of immune-mediated chemosensitivity seems to vary according to the disease setting and BC subtype. As an example, in subgroup analyses of two large randomized immune checkpoint blockade clinical trials, IMPassion130 and KEYNOTE-119, the effect on PFS or OS in the chemotherapy-only arm was the same irrespective PD-L1 status in the locally advanced or metastatic TNBC disease. (163, 164).

In the neoadjuvant setting, a recent meta-analysis reported that pre-treatment PD-L1 expression was associated with increased pCR rates but no subgroup analysis regarding specific subtypes was performed (165). The association between baseline PD-L1 and higher pCR rates has been also demonstrated in prospective-retrospective analysis of two trials on anthracycline-based NACT +/- bevacizumab (67, 166). Moreover, PD-L1 TILs expression predicted response to neoadjuvant trastuzumab emtansine (T-DM1) in the translational study of the phase II

HR+/HER2+ WSG-ADAPT trial (167). Of note, PD-L1 mRNA upregulation was correlated with increased pCR rates in a large retrospective cohort of anthracycline-based NACT (161). Given that PD-L1 expression in immune cells seems to represent a promising favorable prognostic factor in early breast cancer (see paper I), its expression in specific immune cell subpopulations needs to be further explored using spatial pattern approaches (168, 169).

1.13 PD-L1 testing in (breast) cancer: analytical validity, performance and variation in assessment methodologies

The controversial results on the prognostic value of PD-L1 expression in BC, highlight the lack of common evaluation guidelines for the use of PD-L1 as biomarker. Indeed, in the previous reports a plethora of different immunohistochemical antibodies and clones (i.e Ventana: clone SP142, clone SP263; Dako: clone 28-8, clone 22C3), staining platforms, positivity cut-off values and scoring methods have been described according to the compartment of expression, thus hindering the interpretation of the results and implementation in clinical routine. Moreover, PD-L1 evaluation in tissue microarrays and whole tissue sections among the studies underscores the presence of substantial expression heterogeneity.

Following the paradigm of BluePrint projects in lung cancer (170, 171) and other similar approaches (172-174), few studies have directly compared various PD-L1 antibodies in BC (175-177). In a recent multicenter study comparing four clinical developed anti-PD-L1 antibodies in whole tissue sections of TNBC, the PD-L1 immune cell positivity expression (≥1%) was significantly higher for SP263 and similar among the other clones with strong reproducibility and concordance (178). Different antibody clones and scoring systems have been evaluated in another retrospective study including 196 TNBC patients. Similar analytical performance was observed across SP263, 22C3 and 28-8, clones for both tumor and immune cell expression, while lower scores were noted with SP142 (179).

Moreover, the performance of the only approved companion diagnostic PD-L1 Ventana clone SP142 antibody (see below *Biomarkers of immune checkpoint blockade in (breast) cancer and mechanisms of resistance*) has been also investigated in a large retrospective cohort (n=498) of TNBC patients. 46% of the patients were PD-L1 IC positive with heterogeneous staining and correlated with stromal TILs and improved IDFS and OS in the univariate analyses (180).

There is a need of PD-L1 antibody and scoring harmonization (181) for the proper selection of candidate BC patients for treatment with immune checkpoint inhibitors. The different PD-L1 scoring systems, antibody clones and therapeutic monoclonal antibodies are summarized in

Table 1 and challenges are also described below in detail. Scoring methodology consensus and digital pathology/image analysis may provide -as also in the case of TILs- higher accuracy and reproducibility in the evaluation of PD-L1 (182, 183).

Table 1. Comparison of different PD-L1 scoring methods, antibody clones, positivity cut-offs, and correspondence to immune checkpoint inhibitors

Score	PD-L1 scoring description	PD-L1 antibody clone /Company	Cut-off	Checkpoint inhibitor	Trial
IC_A	Percentage of tumor area occupied by PD-L1 stained positive immune cells *or* ***PD-L1+immune cells / tumor area***	SP142 / Ventana (companion diagnostic approved by FDA)	≥1%	Atezolizumab	IMPassion130 KATE-2
CPS	Positive tumor or immune cells as percentage of all tumor cells *or* ***PD-L1+immune cells + PD-L1+tumor cells / tumor cells***	22C3 / Dako	KN-522 ≥1 and ≥10 KN-119 ≥1 and ≥10	Pembrolizumab	KEYNOTE-522 KEYNOTE-119 KEYNOTE-173 KEYNOTE-086 PANACEA TONIC
TPS	Proportion of PD-L1 stained tumor cells over the total number of tumor cells *or* ***PD-L1+tumor cells / tumor cells***	SP263 / Ventana 73-3 / Dako 28-8 / Dako	≥1% (≥5% for any staining and ≥25% for moderate/high staining)	Durvalumab	GeparNuevo
$IC_{IC\%}$	Proportion of PD-L1 stained tumor cells over the total number of immune cells *or* ***PD-L1+ immune cells / immune cells***			Avelumab	JAVELIN

1.14 PD-1 expression in early breast cancer

The expression patterns as well as the prognostic value of PD-1 have not been fully elucidated with results remaining inconclusive due to the lack of standardized methodology in the assessment and variation of study populations. Some previous studies have demonstrated that high PD-1 expression on TILs correlated to worse prognosis (184, 185) while others demonstrated PD-1 was associated with improved survival in TNBC (186-188) especially when expressed in CD8+ T-cells (187). Whether PD-1 gene expression could represent a prognostic marker is yet unclear, even though a report demonstrated promising results in the triple-negative subtype (187), and it merits further investigation in the different BC settings. A comprehensive analysis on the role of PD-1 as a prognostic biomarker in early breast cancer is presented in this doctoral thesis (see paper IV).

1.15 Temporal expression of PD-L1 and PD-1 and prognostic implications in breast cancer

1.15.1 PD-L1/PD-1 expression in primary tumors and in paired metastases

Another topic of interest is the dynamic evaluation of expression of PD-L1 and PD-1 both spatially and temporally. Even though the extent of intra-tumoral heterogeneity of PD-L1 expression and its potential clinical implications have not been evaluated, a growing body of literature is focusing on the expression of these immune checkpoints in the metastatic setting (both lymph node and distant metastases). In a recent study evaluating PD-L1 expression in primary and paired metastatic breast tumors (n=76), both protein and gene expression levels were lower in the metastatic sites (63). Other reports have demonstrated higher levels of PD-L1 expression in the secondary as compared to primary lesions (189, 190). High discrepancies of PD-L1 expression were also noted between primary tumors and distant metastases (28.5% for PD-L1 on tumor and 40.8% for PD-L1 on immune cells), although there was no statistically significant association with survival outcomes (191). Of note in TNBC, PD-L1 expression (immune cells) in distant metastasis did not confer any prognostic value (62) whereas PD-L1 expression in lymph node metastases was correlated to worse disease-free survival (192).

On the other hand, few reports have evaluated the expression of PD-1 in primary and in matched metastatic patient samples. Gene expression of PD-1 was lower in BC metastases versus primary tumors (63). In terms of prognosis, patients with PD-1 protein expression on TILs (23% of the cases) in BC brain metastases were proven to independently correlate with a

favorable outcome (64) while in a recent study with high discordance between primary and matched distant metastases (50% of the patients), association with prognosis was shown (191). Therefore, the additional information and clinical relevance that immune checkpoints can provide remains unknown and more research is needed towards further understanding of the immune microenvironment in BC metastases, given the effect and recent approval of the anti-PD-L1 antibody atezolizumab in patients with triple-negative advanced or metastatic BC. Of note, a post hoc analysis of the IMPassion130 study compared the performance of various PD-L1 IHC assays and demonstrated that PD-L1 expression on immune cells (clone SP142, Ventana, Roche) was higher in the primary as compared to the metastatic tissue (44% versus 36%, p=0.014) and associated with improved PFS and OS in the atezolizumab + nab-paclitaxel arm. Among the different metastatic anatomical locations, PD-L1 was mostly expressed on lymph nodes (51%) and the skin (48%) (193),

1.15.2 PD-L1 / PD-1 expression in the neoadjuvant setting

1.15.2.1 Pre-treatment and post-treatment levels and association with pCR

Given that in the neoadjuvant setting, pre-treatment PD-L1 evaluation or post-treatment was found to be associated with increased rates of pCR in most of the previous studies (194, 195), some recent reports have focused on the evaluation of PD-L1 expression in both the pre- and post-treatment BC samples. In the analysis of 60 patient samples from the SWOG S0800 neoadjuvant trial, levels of PD-L1 protein (tumor or stromal cell) and mRNA expression did not significantly change between pre- and post-treament or between pCR and non-pCR patients (66). In HR+/HER2- breast cancer patients (n=96), there was a trend towards significant decrease in the PD-L1 expression in tumor cells but no association with RCB (67).

1.15.2.2 Breast cancer residual disease

Data on the expression of PD-L1 in the residual disease and its role in the long-term outcomes are scarce. In a study including 309 breast cancer patients with residual disease following neoadjuvant chemotherapy, high PD-L1 expression was independently associated with worse RFS and OS, especially in the triple-negative disease. Moreover, combined PD-L1 high/low TILs (CD8+) was also a factor of adverse outcome (196).

1.16 Effect of chemotherapy on PD-L1/PD-1 axis and immune response

Both preclinical data on different regimens (140) and also clinical data support the effect of chemotherapy on immune response and on the PD-L1/PD-1 immune checkpoint in particular (197). The dynamic expression of PD-L1 and changes in immune infiltration (see above *temporal expression*) might be explained by the immunogenic cell death induces by cytotoxic chemotherapy and that subsequently enables T-cell responses against dying tumor cells (198). The impact of PD-L1 expression upon modulation by NACT in long-term patient outcomes, still remains unclear, even though it can predict pCR. Thus, clinical trials combining chemotherapy and immunotherapy in the neoadjuvant setting as well as post-neoadjuvant chemotherapy in patients who did not achieve pCR are ongoing.

1.17 Immune checkpoint blockade and predictive biomarkers in (breast) cancer

As previously described, immune checkpoints such as PD-1 and its ligand PD-L1 as well as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have been recognized as inhibitory molecules which cause impaired immune response against cancer cells. Further understanding of their role in antitumor immunity has paved the way for the development of immune checkpoint inhibitors, thus revolutionizing the management of a wide spectrum of malignancies (199) and leading to the award of the 2018 Nobel Prize in Physiology or Medicine (https://www.nobelprize.org/prizes/medicine/2018/prize-announcement/).

In a recent network meta-analysis including 11,379 patients (though breast cancer was not included) in 19 randomized clinical trials evaluating the differences between treatment with anti-PD-L1 and anti-PD-1 antibodies, it was shown that anti-PD-1 demonstrated superior OS and PFS as compared to anti-PD-L1. Safety profiles were similar between the two (200).

1.17.1 Immune checkpoint inhibition in breast cancer

In contrast with the plethora of randomized clinical trials published in various tumor types, prospective randomized data in BC using immune checkpoint inhibitors currently come from a few major clinical trials (201) which are summarized in Table 2.

Metastatic setting: The IMPassion130 is the only randomized phase 3 trial which demonstrated benefit from the combination of atezolizumab and nab-paclitaxel compared to nab-paclitaxel alone in patients with triple-negative metastatic BC and PD-L1 immune cell positivity (163, 202). In the intention-to-treat population (ITT) (n=902), median PFS (7.2 versus 5.5 months)

but not OS (21.3 versus 17.6 months, non-significant) was statistically significantly prolonged with the addition of atezolizumab to chemotherapy. In the PD-L1 immune cell-positive cases treated with atezolizumab, median PFS remained improved (7.5 versus 5.5 months; HR= 0.62, 95% CI 0.49-0.78, p<0.001) and importantly OS was also prolonged (25 versus 18 months; HR=0.71, 95% CI 0.54-0.94) in this patient subgroup as shown in the updated overall survival data (202). Based on this study results, Federal Food and Drug Administration (FDA) has approved the use of the first immune checkpoint inhibitor in BC patients in 2019 (203).

Apart from the combination of immune checkpoint inhibitors with chemotherapy, recent data from pembrolizumab monotherapy in patients with metastatic TNBC treated with prior chemotherapy, have been reported in the KEYNOTE-086 phase 2 study. In cohorts A (n=170, PD-L1 unselected patients) and B (n=84, only PD-L1 positive tumors included), the objective response rates (ORR) were 5.3% and 21.4%, respectively (204, 205). On the other hand, the phase 3 KEYNOTE-119 did not meet its primary endpoint for improved OS in the pembrolizumab arm as compared to chemotherapy in metastatic TNBC (164).

Moreover, according to the preliminary press release of the randomized phase III KEYNOTE-355 trial (ClinicalTrials.gov, NCT02819518), the addition of pembrolizumab to chemotherapy indicated statistically significantly improved PFS in PD-L1+ (CPS ≥10) metastatic TNBC patients as compared to chemotherapy alone, with the announcement of the results to be awaited (news release by Kenilworth, NJ: Merck; https://bit.ly/2HtT4rj).

Finally, in the whole cohort of 67 TNBC patients receiving nivolumab in combination with chemotherapy or radiotherapy, the ORR was 20% with the majority of responses observed in the cisplatin and doxorubicin arms, as demonstrated in the adaptive phase II TONIC trial (206). Of note, the increased TILs, PD-1 (in immune cells) and immune-related genes in responders versus non-responders indicated that treatment can induce an inflamed tumor microenvironment which can further sensitize patients to PD-1 blockade.

Neoadjuvant setting: In the phase 3 randomized trial KEYNOTE-522, neoadjuvant pembrolizumab in combination with chemotherapy resulted in significantly increased pCR rates as compared to chemotherapy alone (64.8% versus 51.2%, respectively), regardless of PD-L1 status (207). Furthermore, addition of pembrolizumab to chemotherapy resulted in a pCR rate of 60% (range 30-80%) and prolonged 12-month EFS (80-100%) across all cohorts in the recently reported neoadjuvant KEYNOTE-173 trial (208).

Recent results from the ongoing open-label, multicenter, adaptively randomized phase 2 I-SPY2 platform trial including 69 patients randomized to pembrolizumab + chemotherapy versus chemotherapy alone (n=181) indicated improved pCR rates for the ICB arm (44%

versus 17% in ERBB2-negative, 30% versus 13% in HR-positive/ERBB2-negative and 60% versus 22% in TNBC patients) while achievement of pCR was also predictive of EFS, especially for patients in the pembrolizumab arm (209). The addition of the anti-PDL-1 monoclonal antibody durvalumab to chemotherapy was associated with increased pCR rates (61.0% for durvalumab versus 41.4%, for chemotherapy alone) as shown in the randomized phase 2 GeparNuevo trial, comparing durvalumab + chemotherapy versus placebo + chemotherapy as a neoadjuvant treatment in TNBC patients (210).

On the other hand, preliminary results of the NeoTRIPaPDL1 Michelangelo randomized trial allocating 280 TNBC patients to neoadjuvant chemotherapy +/- atezolizumab did not result in statistically significant differences in pCR rates neither in the intention-to-treat population (43.5% for atezolizumab vs 40.8% for chemo only) nor in the PD-L1+ subgroup (51.9% for atezolizumab vs 48.0% for chemo only) (211).

Since, new therapeutic agents that stimulate a sufficient immune effect have been introduced into clinical practice, offering a better clinical outcome in cancer patients, several trials using anti-PD-1/PD-L1 antibodies (201) as well as other immunomodulatory agents are ongoing in different BC disease settings (neoadjuvant, adjuvant, metastatic) with the results to be eagerly awaited. Moreover, combinational strategies including immune checkpoint inhibitors and chemotherapy and/or targeted therapy (i.e HER-2 targeted treatment: KATE-2 phase II trial, enrolling 202 advanced BC patients to T-DM1 +/- atezolizumab, suggesting a OS benefit for PD-L1+ patients in the atezolizumab arm; the PANACEA phase Ib/II trial including 58 advanced BC patients to single arm pembrolizumab +trastuzumab, demonstrating an objective response of 15% among PD-L1+ patients (212, 213)) have been reported or are currently under investigation so as the optimal partner which can enhance immunotherapy efficacy to be identified (214, 215).

1.17.2 Biomarkers of immune checkpoint blockade in (breast) cancer

i. PD-L1 IHC expression

Research efforts have been made for the identification of reliable predictive biomarkers for immune checkpoints inhibitors in various cancer types (140, 216). PD-L1 expression has been explored as a predictive marker in a large across cancer meta-analysis (n=4174; BC trials were not included) but with inconclusive results (OS HR=0.66, 95% CI 0.59-0.74 for PD-L1+ and OS HR=0.80, 95% CI 0.71-0.90 for PD-L1- patients) (217). Its role in BC is also currently under investigation (218). In a secondary analysis of IMPassion130, it has been recently reported that PD-L1 expression ≥1% in immune cells (SP142 clone, Ventana) was a predictor

of treatment benefit with the combination of atezolizumab and nab-paclitaxel (219). Similar associations were seen between PD-L1 expression in immune cells and both longer ORR and overall survival in patients with metastatic TNBC treated with atezolizumab monotherapy according to the results of a recent phase 1 study (220). The relative advantage of the report of many studies where PD-L1 expression is correlated with increased efficacy is counterbalanced by the observed discrepancies among the current scoring systems and methodologies, antibody clones, cut-off values for the various monoclonal antibodies which have been described above (Table 1). Moreover, the spatial heterogeneity of PD-L1 expression further hinders its concordant and reproducible evaluation (221).

Several retrospective results on prospective clinical trials have tried to identify the optimal scoring of PD-L1 protein. In a post-hoc analysis (n=614) of the IMPassion130 trial, the analytical concordance of three different PD-L1 antibody clones (IC: Ventana SP142, Ventana SP263 and CPS: Dako 22C3) was explored. SP263 and 22C3 identified more patients with PD-L1 positivity as compared to SP142. However, in PD-L1+ patients the evaluation with the SP142 antibody predicted benefit regardless of positivity or negativity in other antibodies (193). Furthermore, in the other metastatic phase III KEYNOTE-119 study it was shown that pembrolizumab correlated with improved efficacy (OS HR=0.58 and PFS HR=0.76) in TNBC patients with high PD-L1 expression (CPS≥20) and durable responses as compared to chemotherapy (164).

Of note, neoadjuvant pembrolizumab in combination with chemotherapy demonstrated statistically significantly increased pCR rates as compared to chemotherapy alone regardless of PD-L1 status in TNBC patients as shown in the KEYNOTE-522 study (207). In contrast, the neoadjuvant phase Ib KEYNOTE-173 study PD-L1 CPS was significantly correlated with improved response to anti-PD-1 pembrolizumab treatment (208). In addition, in the phase II GeparNuevo trial, PD-L1 expression in tumor cells was significantly associated with increased pCR in the durvalumab arm (210). The predictive capacity PD-L1 IHC expression as demonstrated in the context of breast cancer ICB clinical trials is also presented in Table 2.

ii. Tumor-infiltrating lymphocytes

The role of sTILs expression as predictors of immunotherapy response has been examined in the context of some prospective clinical trials (Table 2). In the metastatic setting patients with high sTILs numbers were associated with increased response to pembrolizumab as compared to chemotherapy both in the KEYNOTE-086 (204, 205) and KEYNOTE-119 (TILs ≥5%; OS HR= 0.75 (95% CI, 0.59-0.96)) (222) trials. Moreover, in the biomarker analysis study of

IMPassion130 (219), patients with either stromal TILs enrichment or CD8+ tumors demonstrated improved PFS and OS only when PD-L1 was simultaneously expressed on immune cells.

Similarly, in the neoadjuvant setting, in the phase Ib KEYNOTE-173 study, higher baseline and on-treatment sTILs predicted for increased pCR rates in patients treated with neoadjuvant pembrolizumab and chemotherapy (208), while in both KEYNOTE-522 and in GeparNuevo higher sTILs predicted for higher pCR rates in both arms (207, 210).

iii. Immune-related genes and signatures

The role of 18 immune-related gene signatures as predictors to immunotherapy has been investigated in TCGA and in two retrospective cohorts (n=177 and n=65) and their expression (especially those related T-cell and IFN-γ) were associated with better anti-PD-1 response (223-225). Of note, at the single gene level, PD-1 was strongly correlated with immune gene signatures, CD8 T cell genes and also with increased anti-PD-1 response rates, thus outperforming other markers (224). Since data come from only a single study including very few BC patients (n=3), PD-1 mRNA value needs to be further validated in a prospective trial.

Furthermore, in the translational biomarker sub-study of the prospective GeparNuevo trial, IFN-gamma signatures were associated with increased pCR rates in TNBC patients treated with the neoadjuvant anti-PD-L1 antibody durvalumab while TILs signature was predictive for response in both arms (226).

iv. Tumor mutational burden

The clinical utility of high tumor mutational burden (TMB) as a predictive biomarker to PD-1 inhibition has been retrospectively (227) and prospectively validated in a randomized trial, demonstrating association with treatment benefit regardless of PD-L1 expression (228). Despite its promising capacity as a biomarker, its implementation in routine practice can be challenging (229, 230). In breast cancer, continuous TMB significantly independently predicted pCR (OR=2.06, 95%-CI: 1.33-3.20, p=0.0012) but did not predict effect of durvalumab (231).

v. Other predictive biomarkers

In hematological malignancies, PD-L1 gene amplification was correlated with high response rates (141), but amplification seems to be a rare event in solid tumors (142). Specific mutations and genetic polymorphisms can also influence immune checkpoint treatment response and merit further investigation (216). Moreover, a recent large meta-analysis for the investigation of predictive biomarkers to immune checkpoint blockade including a variety of methods, revealed the promising role of multiplex IHC over previously characterized markers (232). Thus, their clinical utility should be further validated in prospective clinical trials and the underlying resistance mechanisms and TIME interactions (233) should be thoroughly studied in order novel promising biomarkers to be discovered.

Drug(s)	Phase (subtype)	Number of patients	Treatment arms	Primary endpoint (survival outcome, pCR, ORR)	Predictive biomarker analyses	
					PD-L1	TILs
Metastatic/Advanced setting						
IMPassion 130	III (TNBC)	902	Nab-paclitaxel +/- atezolizumab	ITT: PFS: 7.2 versus 5.5 months; HR=0.80, 95% CI 0.69-0.92, p=0.002); OS (last update Jan 2, 2019): 21 versus 18.7 months; HR=0.86, 95% CI 0.72-1.02; p=0.078)	Correlation of PD-L1 positive immune cell expression (≥1%; SP142 clone) with improved PFS (HR 0.62. CI 95% 0.49-0.78) and OS (HR 0.62, CI 95% 0.45-0.86)	Correlation of TILs expression levels (>10%) with better PFS (HR=0.53, CI 95% 0.38-0.74) and OS (HR=0.57, CI 95% 0.35-0.92) in the PD-L1+ population (n=460)
KEYNOTE-086 (cohort A: PD-L1 unselected patients)	II (TNBC)	170	Pembrolizumab monotherapy vs chemotherapy	Total ORR = 5.3%	No significant correlation of PD-L1 positive tumors (CPS≥1; 22C3 clone) with improved ORR (5.7% vs 4.7%) and no differences in OS and PFS	Correlation of high TILs expression (>median) with increased response to pembrolizumab (ORR 6% vs 2%) and higher TILs levels in responders versus non-responders (10% vs 5%)
KEYNOTE-086 (cohort B: PD-L1 positive patients)	II (TNBC)	84	Pembrolizumab monotherapy vs chemotherapy	Total ORR = 21.4%	PD-L1 was a predefined marker of patient selection	Correlation of high TILs expression (>median 17.5%) with increased response to pembrolizumab (ORR 39% vs 9%) and higher TILs levels in responders versus non-responders (50% vs 15%)

KEYNOTE-119	III (TNBC)	622	Pembrolizumab monotherapy vs single-agent chemotherapy (physician's choice)	ITT: No significant differences in terms of PFS (HR=1.60 CI 95% 1.33-1.92), OS (HR=0.97 CI 95% 0.82-1.15) and ORR (9.6% vs 10.6%)	Pembrolizumab correlated with improved efficacy OS HR=0.58 CI 95% 0.38-0.88; and PFS HR=0.76, CI 95% 0.49-1.18) in TNBC patients only with high PD-L1 expression (CPS≥20; 22C3 clone)	Correlation of TILs expression levels (≥5%) with improved OS (HR= 0.75, 95% CI 0.59-0.96) only in patients treated in the pembrolizumab arm
TONIC	II (TNBC)	67	Chemotherapy or radiation therapy (induction) +/- nivolumab	Overall cohort: ORR=20% ORR: Cisplatin=23% and Doxorubicin= 35%	Correlation of PD-L1 positivity in immune cells (≥5%; 22C3 clone) and not in tumor cells (≥1%) with better ODS and OS	Responders had significantly higher sTILs as compared to non-responders (median 12.5% vs 6%)
Neoadjuvant setting						
KEYNOTE-173	Ib (TNBC)	60	Pembrolizumab + chemotherapy	pCR rates = 60% (range 30-80%) and 12-month EFS = 80-100% across all cohorts	Correlation of PD-L1 (CPS≥1; 22C3 clone) with increased pCR	Significant correlation of higher sTILs (baseline and on-treament) with increased pCR. Higher baseline and on-treatment TILs in responders
KEYNOTE-522	III (TNBC)	1174	Pembrolizumab + chemotherapy vs chemotherapy	Higher pCR rates with the addition of pembrolizumab (64.8% versus 51.2%)	No correlation of PD-L1 (CPS≥1; 22C3 clone) with pCR	Correlation of higher sTILs with increased pCR

GeparNuevo	II (TNBC)	174 (window cohort: 117)	Durvalumab + chemotherapy vs chemotherapy	pCR = 61.0% for durvalumab versus 41.4%, for chemotherapy and OR = 2.22, 95% CI 1.06-4.64, P = 0.035 ; only in the window cohort	Correlation of PD-L1+ tumor cell expression with increased pCR in the durvalumab arm	Correlation of higher sTILs with increased pCR in both arms

Abbreviations: TNBC, triple-negative breast cancer; pCR, pathologic complete response; ORR, objective response rate; ITT, intention-to-treat; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; PD-L1, programmed death ligand 1; TILs, tumor-infiltrating lymphocytes; OR, odds ratio; EFS, event-free survival; CPS, combined positive score

Table 2. Overview of major published phase II/III clinical trials targeting PD-L1/PD-1 in breast cancer with biomarker analyses

2 AIMS OF THE THESIS

The overall purpose of this project was to elucidate the expression, regulation and prognostic role of immune infiltrate and the Programmed Death-1/ Programmed Death Ligand 1 (PD-1/PD-L1) checkpoints in early and metastatic BC.

The specific aims of the study were:

Aim 1: To determine the expression of PD-L1 and PD-1 and their prognostic significance in early BC

Goal 1. To evaluate the protein expression of PD-L1 and PD-1 in tumor and/or immune cells and investigate the associations with patient characteristics in different subtypes.

Goal 2. To investigate the prognostic capacity of PD-L1 and PD-1 gene expression and correlation with validated genomic signatures

Aim 2: To characterize the immune infiltrate and explore the prognostic and predictive role of its components in early, non-metastatic BC

Goal 1. To evaluate TILs and other immune cell subtypes (e.g. tumor-associated macrophages) and explore their clinical correlations.

Goal 2. To explore the interplay of immune cell subpopulations with PD-L1 expression using indirect *in silico* information provided by gene expression profiling.

Aim 3: To investigate the mechanisms driving lymphocytic infiltration and PD-L1 regulation in BC

Goal 1. To investigate the regulatory mechanisms of PD-L1 *in vitro* and *in vivo*

Goal 2. To explore the impact of PD-L1 regulation and immune cell infiltration in human breast tumors

Aim 4: To evaluate the predictive value of immune-related components in metastatic breast cancer patients receiving chemotherapy

Goal 1. To explore the predictive value of immune infiltration to chemotherapy-treated metastatic BC patients

Goal 2. To determine the predictive role of immune-related genes and signatures for the effect of chemotherapy in the metastatic BC setting

3 PATIENT AND METHODS

3.1 Patient populations

i. The "Merck" early breast cancer cohort *(papers I-IV)*

This patient cohort consisted of 646 women patients diagnosed with primary BC between 1997 and 2005 in health care region of Stockholm, Sweden that were retrospectively selected using the Stockholm-Gotland Breast Cancer Registry. The patient selection was initially based on a nested case-control design (cases: patients developing distant metastatic disease; controls: patients free from distant disease, matched by adjuvant therapy, age and calendar period at diagnosis) but a direct cohort approach was adopted for the scope of the present studies (also previously described (234)). Moreover, this cohort included patients (n=59) treated with neoadjuvant chemotherapy, who were excluded from further analyses whenever indicated and accordingly depicted in the respective flowcharts. Data on clinical and pathological tumor characteristics, survival and loco-regional/systemic treatments have been collected and previously reported (234). Distant metastasis-free interval (DMFI), defined as the period of time from date of diagnosis to the first evidence of distant metastasis (Distant recurrence-free interval: DRFI, if death from BC was also included) and overall survival (OS), defined as the period of time from date of diagnosis to death of any cause -both censored after 15 years- were used as clinical endpoints. The reverse Kaplan–Meier estimate (235) of the median DMFI and OS follow-up was 12.4 years (up to December 31, 2012) and 15 years (up until January 10, 2015), respectively. Furthermore, Biospecimen Reporting for Improved Study Quality (BRISQ) criteria for this cohort have been previously published (234). Karolinska Institute's (Stockholm, Sweden) ethics committee has approved all the performed analyses (included in papers I - IV) in this patient cohort (Dnr 2006/394-31/3, 2006/1183-31/2 and amendments 2016/1505-32, 2018/789-32, 2018/790-32) and decided that no additional informed consent from the patients was needed.

ii. The Cancer Genome Atlas (TCGA) breast cancer publicly available dataset *(papers I-IV)*

The Cancer Genome Atlas (TCGA) provisional dataset (236), consisting of 1,100 patients with primary breast cancer and available RNA-sequencing (RNA-seq) and clinical-pathological data was used for various analyses (included in papers I – IV). The publicly available data generated by the by the TCGA Research Network (https://www.cancer.gov/tcga) were

retrieved from cBioportal (237, 238) on November 21st, 2018. OS and Progression-free interval (PFI) (defined as the time period from the date of diagnosis to the date of the first occurrence of a new tumor event, including progression of the disease, locoregional recurrence, distant metastasis, new primary tumor, or death with disease) were used as clinical endpoints as recommended; PFI was extracted from the standardized TCGA Pan-Cancer Clinical Data Resource dataset (236). Median follow-up for this cohort (reverse KM estimate) was 2.5 for PFI and 2.6 years OS, both censored after 10 years.

iii. The TEX metastatic randomized clinical trial *(paper V)*

TEX was a multicenter, randomized phase III trial (ClinicalTrials.gov identifier NCT01433614) evaluating the activity of epirubicin and paclitaxel, with or without capecitabine, as first line treatment for locally advanced inoperable or metastatic breast cancer (239). In total, 287 patients were randomized and tailored treatment doses were used depending on the related side effects. A third treatment group with fluorouracil, epirubicin and cyclophosphamide (FEC) was closed shortly after the initiation of the study. In light of the publication of data regarding trastuzumab efficacy, the initially allowed enrollment of patients with HER2 positive disease was discontinued. For the translational part of this study see below (paragraph: *GEP data derived from original study cohorts*). Radiological tumor assessments were performed according to the protocol after every third 3-week chemotherapy cycle and evaluated according to the Response Evaluation Criteria In Solid Tumors (RECIST) version 1.0. The endpoints used included: i) Best objective response and ii) size change of target lesions as documented and centrally reviewed for each patient, iii) progression-free survival (PFS), defined as the interval from date of randomization to date of disease progression or death and iv) time to treatment failure (TTF), defined as the period from the date of randomization to that of end of study treatment for any reason (disease progression, patient's choice, treatment toxicity or death). Both Karolinska Institute's (Stockholm, Sweden) ethics committee (Dnr 02-206) and Swedish Medical Product Agency have approved the clinical study and all the performed correlative analyses (included in paper V) for all participating centers. All patients have provided informed consent for their participation in the study.

3.2 Gene expression profiling (GEP): RNA isolation, data generation, preprocessing, normalization and analysis

A) GEP data derived from original study cohorts

i. The "Merck" early breast cancer cohort

Total RNA was extracted from primary fresh frozen tumor tissue of all patients, using the Qiagen RNeasy Mini Kit, (Qiagen, Germany), and gene expression profiling was performed using NuGEN amplification protocol. Samples were then hybridized using the Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarray (NCBI GEO depository as GPL10379). Details about the experimental methods and microarray data (included in papers I-IV) are available at the Gene Expression Omnibus database, under the accession number GSE48091). In order to obtain a log-transformed expression value for each probe set, the raw GEP data were background corrected, normalized and summarized using the robust multichip averaging (RMA) (240) method implemented in the aroma.affymetrix R package (241). A nonspecific filter was used and in the case of multiple mappings to the same Entrez Gene ID, the probe sets with the highest interquartile range were chosen. The preprocessing and normalization of the GEP data were performed using R computing environment.

ii. TEX metastatic trial

As part of the translational aspect of the trial, metastatic biopsies were obtained mostly by fine-needle aspiration biopsies (FNAB) (97.6%) or core biopsy (2.4%), in a total of 149 patients. In these patients, a biopsy from at least one metastatic site was taken before treatment start as follows: lymph nodes = 36.7%, liver = 22.5% skin = 18.3%, breast other sites = 6.7%. of the patients. RNA was subsequently extracted and its integrity was evaluated using the Agilent 2100 Bioanalyzer (28S: 18S RNA ration). Samples were then profiled and hybridized using NuGEN protocol and Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarray (GEO: GPL10379), respectively (242). Further details regarding GEP data are available at the Gene Expression Omnibus database under accession number GSE56493. Before the differential expression analysis, a variance filter was employed (probes with a SD < the median SD for all probes were excluded) so as uninformative probe sets to be removed (242).

iii. GEP data derived from The Cancer Genome Atlas (TCGA)

The RNA sequencing data (RNA-seq v2 RSEM) for the evaluation of mRNA expression in the Provisional TCGA dataset (included in papers II and III), were downloaded from cBioportal (237, 238). These data (level 3) have been produced after i) alignment of the raw reads to the human h19 genome assembly using MapSplice (243), ii) quantitation at the gene (and isoform)

level using RSEM (244), and iii) application of upper-quartile normalization (245). In order to transfrom the normalized retrieved data to log2-expression, 1 was added to each value.

iv. GEP data derived from pooled publicly available transcriptomic datasets

A pooled gene expression analysis from thirty-nine publicly available datasets including transcriptomic profiles of more than 9500 primary breast cancers (papers I and IV for PD-L1 and PD-1 gene expression, respectively) was performed. Gene expression data from the 38 previously described public databases (246, 247) were retrieved and data from the Merck adjuvant cohort (GEO:GSE48091) (see above: *patient populations* and *Gene expression profiling*) were also added. In order for comparability of expression values across multiple datasets to be ensured, a robust linear scaling was applied to each gene such that expression quantiles 2.5% and 97.5% were set to −1 and +1, respectively. Due to incomplete information about ER and HER2 status among the datasets, ESR1 and ERBB2 status were classified based on the bimodal distribution of their expression values.

3.3 Gene expression signatures

i. PAM50 intrinsic subtypes *(papers II, IV and V)*

The intrinsic molecular subtyping was assigned to each tumor using the research-based 50-gene subtype predictor (PAM50) (248) and applied across the cohorts.

Notably in paper II, a similar to the iterative approaches (249, 250) was used due to ER status discrepancies. In brief, GEP were matched to those derived from the original ER distribution in the original PAM50 report (248), in order an ER balanced subset to be formed. The whole dataset was median centred (as recommended in (251, 252)) and assigned to one of the intrinsic molecular subtypes. Samples with Spearman's rank correlation coefficient < 0.1 were considered as not classified (250). More details can be found in the respective methods section of paper II

Given that PAM50 signature was developed in a primary breast cancer cohort, as described in the original publication (248), the distribution of standard clinico-pathological markers (e.g ER, PR, HER2, nodal status, grade and size) was typical for the primary tumor setting. In contrast, the biological distribution is different in our TEX metastatic cohort material (presented in paper V) which includes tumors with more aggressive characteristics (e.g. ER-negative and highly proliferative tumors). Therefore, the derived gene expression arrays were normalized with those derived from the primary breast cancer adjuvant Merck cohort (see above), profiled with the same platform (242). Subsequent PAM50 subtyping was accordingly

performed (248) upon normalization and median centering across genes. Of note, both in this study and in the pooled gene expression datasets (used in papers I and IV), the normal breast-like tumors were not included in the subgroup analyses, due to the lack of a clear established consensus on the existence of this subtype.
Furthermore, for independent confirmation of the intrinsic subtyping the Absolute Intrinsic Molecular Subtyping (AIMS) approach was applied in the TEX trial (253).

ii. 21-gene and 70-gene signatures *(paper II)*

The research-based signature scores and risk classifications (used in paper II) were computed based on the originally described signatures for 21-gene (commercially known as OncotypeDx) (254) and 70-gene (commercially known as Mammaprint) (255), upon implementation in the *genefu* R/Bioconductor package (256). The procedure of signature application included median centering of the available GEP data and mapping of genes for both signatures through Entrez Gene IDs. The 21-gene signature included 5 reference genes that were not used in gene signature calculations while in the 70-gene signature there were 14 probes with unknown Entrez Gene IDs/ HUGO gene symbols and a couple of other probes which are missing from the platform (*see* paper II for gene availability in each cohort).

iii. Phosphorylated STAT3-Associated Gene Signature *(paper III)*

The phosphorylated STAT3-associated gene signature (pSTAT3-GS) was originally generated to predict trastuzumab resistance and shown to predict the proteomic pSTAT3 activation status (257). The provided pSTAT3-GS's gene symbols were converted to Entrez Gene IDs using DAVID's Gene ID Conversion Tool (258), and mapped both to the Merck's and TCGA breast cancer cohorts microarray's probe sets (paper III). The continuous signature scores, i.e., signed averages, were calculated using the genefu R package (256). In total, out of the 123 differentially expressed genes, 114 and 122 were mapped in Merck and TCGA datasets, respectively and subsequently used for the signature calculation.

iv. Immune-related and other gene signatures *(paper V)*

The predictive value of six gene expression signatures was explored in advanced breast cancer chemotherapy treated patients in the TEX trial (paper V), including: i) immune-related (48, 121), ii) proliferation-related (259), iii) ER-related (260) and iv) the molecular aberrations-related gene signatures for PIK3CA (261) and TP53 mutations (262). The weighted averages

(+1 or -1) of the expression values of the constituent signature genes were used for the calculation of the gene module scores (named as Immune module 1 and 2 (48), Proliferation, ESR1, PIK3CA and TP53 modules, respectively). A non-specific filter was first applied to the gene expression data and only original probe-sets or genes that could be mapped to Entrez Gene IDs where included in further analyses. In case of multiple probe-set mappings to the same Entrez Gene ID, those with the highest interquartile range were chosen.

3.4 Bioinformatic tools for *in silico* analysis of immune cell subpopulations and TME

i. CIBERSORT *(paper II)*

For the further characterization and quantification/deconvolution of immune cell subpopulations using GEP-based data in the Merck cohort (paper II), the *in silico* CIBERSORT method (R script version 1.04) was employed (110). This computational approach and its gene signature matrix LM22 containing 547 genes, can distinguish 22 hematopoietic cell phenotypes from a bulk tumor tissue biopsy (263). Each patient in our cohort generated an absolute immune fraction score (median expression level of all genes in the signature matrix/median expression level of all genes in the mixture), with 100 permutations to be selected as a default.

ii. Gene set enrichment analysis *(paper V)*

In order to compare the differential expression of genes corresponding to important biological procedures between chemotherapy responders and non-responders in the TEX metastatic trial (paper V), gene set enrichment analysis (GSEA) of the Reactome gene sets collection in the Molecular Signatures Database (MSigDB, Broad Institute, version 5.2) was performed. The GSEA Software (Broad Institute, version 2.2.3) (115) was used and genes were pre-ranked through the R/Bioconductor package limma (version 3.30.4) (264).

iii. Microenvironment Cell Populations counter *(paper V)*

For the quantification and estimation of the absolute abundance of eight immune and two stromal cell populations derived from GEP data, the Microenvironment Cell Populations counter (MCP-counter) method was performed using the R package MCPcounter (version 1.1.0) (112) in the same cohort (TEX trial; paper V)

3.5 Breast cancer tissue preparation and staining methods

i. Tissue microarrays (TMAs) construction (papers I, II, IV)

Tissue microarrays (TMAs) have been constructed from representative tumor-rich areas from all primary tumors in the Merck cohort, using an automated tissue microarrayer (VTA-100, Veridiam, San Diego, CA, USA). Each TMA consisted of duplicate per tumor and each core had a diameter of 1 mm.

ii. Whole tissue sections from FFPE breast cancer tissue and cell lines (paper III)

Whole tissue sections (4 um) were prepared from formalin-fixed paraffin-embedded (FFPE) tissue blocks for a subset of patients in Merck cohort according to the expression of PD-L1 in tumor cells (positive and negative). Furthermore, cell pellets from cultured breast cancer cell lines (MCF7, MDA-MB-231, BT549, *see below*) were collected, fixed in formalin and embedded in paraffin to prepare FFPE cell blocks and subsequent sections.

iii. Immunohistochemistry (IHC) (papers I-IV)

Tissue sections (4um) from the constructed TMAs were prepared and used for IHC. Immunostaining with the primary monoclonal antibodies targeting PD-L1 (clone SP263, Ventana, Roche; papers II and III), CD3 (clone 2GV6, Ventana, Roche; paper II) and PD-1 (clone NAT105, Ventana, Roche; paper IV) were performed using the Ventana auto-stainer (Roche, Basel, Switzerland) according to the manufacturer's protocol. Reactive lymphoid tissue of the tonsil was used as a control for both PD-L1 and CD3 IHC included.

In FFPE sections of breast cancer cell line tissue blocks, IHC was manually performed using anti-phosphorylated STAT3/anti-pSTAT3^{Y705} (clone B-7. Santa Cruz Biotechnology), anti-STAT3 (clone 79D7, Cell Signaling Technology) and anti-PD-L1 antibodies (clone EL1N3, Cell Signaling Technology), as briefly follows: i) slides' deparaffinization and rehydration, ii) antigen epitope retrieval, iii) peroxide and protein blocking, iv) immunostaining with primary antibody (overnight incubation), v) primary antibody binding detection with a HRP-coupled polymer combined with DAB chromogen, vi) counterstaining with hematoxylin, vii) de-hydration with gradient ethanol concentrations and viii) mounting. The anaplastic large lymphoma cell line Mac2A was used as a positive control for all these antibodies. Immunostaining with anti-pSTAT3^{Y705} (clone B-7. Santa Cruz Biotechnology) was also performed on whole tissue sections in a subset (n=83) of patients in the Merck cohort (paper III). Moreover, ER, PR (collected from pathology reports), HER2 (central evaluation using

CISH) and Ki67 immunohistochemical staining and evaluation methods have been previously described in the Merck cohort (234). Sice ER, PR and HER2 positivity (either via IHC or FISH) was challenging to be defined in TCGA (265), the reported statuses of the biomarker were used as described in the original publication (236).

iv. Immunofluorescence (paper III)

For the evaluation of macrophage markers' expression in whole-tissue sections FFPE breast cancer patient tissue samples, double IF was performed as follows: i) antigen retrieval with sodium citrate buffer, ii) blocking using buffer containing PBS, 0.3% Triton X-100, 10% FBS and 1% BSA, iii) immunostaining with anti-CD163 (Leica) and anti-CD11c (Leica) antibodies and incubation with AlexaFluor 488 or 546 fluorochrome-conjugated secondary antibodies (Life Technologies) while cell nuclei were labeled with DAPI (Invitrogen Corp.). Binding specificity was ensured using isotype specific antibodies. For details regarding all antibodies see paper III (266).

v. Hematoxylin and Eosin (H&E) staining *(paper V)*

In order to evaluate the extent of lymphocytic infiltration in the advanced breast cancer setting, FNAB smears were stained by standard H&E (or Giemsa) staining protocols.

vi. Fluorescence in Situ Hybridization (paper III)

The CD274 gene locus status was analyzed on FFPE breast cancer cell pellets using FISH with the probe and protocols as recommended by the manufacturer (ZytoVision, GmbH). The validated CD274 probe expression (covering 9p24.1 gene locus) was quantified as copies under a fluorecescent microscope with a centromeric chromosome 9 probe as control. The ratio CD274/CEP9 ≥2 was considered as amplification (267-269).The Hodgkin lymphoma cell line HDML2 was used as positive control for CD274 amplification.

3.6 Image acquisition, analysis and scoring methods

i. Tissue microarrays *(papers II and IV)*

PD-L1: PD-L1 was evaluated in tumor, immune cells and/or both cell types (total cell expression). PD-L1 positivity was defined by the presence of any single cell with membranous PD-L1 immunostaining (tumor or immune respectively). A TMA core was considered as PD-

L1+ when at least one cell expressed the marker. In case of discordant expression between duplicate TMA cores, the positive one was selected

CD3: CD3 IHC-stained TMA slides were digitally scanned (NanoZoomer-XR, Hamamatsu Photonics K.K, Japan) and manual scoring of the total number of CD3 positive (CD3+) cells was performed using ImageJ software v. 1.48 (NIH, Bethedsa, MD, USA). CD3+ membranous staining lymphocytes were counted in each TMA core and the average number was calculated over the duplicates (average number of CD3+ cells per tumor sample).

PD-1: The total number of PD-1 positive cells, defined as the presence of any membranous immune cell staining, was manually counted under the microscope for each TMA core. The average number of PD-1+ positive cells was then calculated between the duplicate cores, thus representing the PD-1+ immune cells score per tumor sample.

For all the aforementioned markers, the evaluation was performed by two independent investigators including a certified pathologist, while TMA cores with poor staining quality, limited or missing tumor, folded tissue were -upon discussion- excluded from the analysis.

ii. Whole tissue sections *(paper III)*

pSTAT3 IHC: Five different high power fields were selected per tissue section. At least 300 tumor cells were manually counted using ImageJ software (NIH, Bethesda, MD, USA) and the percentage of pSTAT3 positive cells (nuclear expression) was subsequently calculated across the selected areas.

Macrophage markers (CD163, CD11c) IF: Eight independent fields from each tumor section were analyzed using LSM T-PMT Zeiss confocal microscope. The abundance of positive cells over the total cell number was quantified per area using ImageJ software (NIH, Bethesda, MD, USA) and the average percentage of expression per marker was calculated across the selected areas.

iii. Fine-needle aspiration biopsies *(paper V)*

Lymphocytes on FNAB: For the quantitative assessment of immune infiltrate, an algorithm for lymphocyte counting in FNAB smears was developed. A minimum of 10 cancer-cell clusters containing at least 10 cells each were evaluated per smear. In each cluster, total number tumor cells and lymphocytes were separately enumerated and the average percentage of lymphocytes over total cells was calculated for each patient sample.

3.7 In vitro studies and laboratory techniques

i. Cell Lines and culturing conditions *(paper III)*

A number of cell lines were used in this study for the mechanistic in vitro studies including: a) the human breast cancer cell lines MCF7 and MDA-MB-231, BT549 and SKBR3, b) the mouse mammary carcinoma cell line 4T1, c) the anaplastic large cell lymphoma Mac2A cell line and d) the Hodgkin lymphoma cell line HDLM2. The cell lines were grown in complete RPMI-1640 or DMEM media (Gibco/Life Technologies) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin and at 37 ◦C in 5% CO2 according to ATCC protocols.

ii. Pharmacologic inhibition, transient transfections and gene silencing *(paper III)*

Pharmacologic treatment on breast cancer cells was performed using the selective STAT3 SH2 domain binder inhibitor C188-9 (Calbiochem) and recombinant human IL-6 (PeproTech) at indicated concentrations for 48h and 24h, respectively.

Cells were seeded at a density of 0.2–0.6 × 106 cells/ml 24 h before prior transfection. The BT549 breast cancer cell line was transfected with siRNA oligonucleotide (300 nM) specific for the gene sequence of STAT3 and control siRNA (All Stars Negative, QIAGEN) using Lipofectamine 2000 (Thermofisher) reagent per protocol. The transfected SKBR3 breast cancer cells with vector control and STAT3C-plasmid (kindly provided by Drs Sarah Walker and David Frank, Department of Medical Oncology, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, MA, USA). Whole cell lysates of the treated and transfected cells were then prepared for western blot analysis.

The 4T1 mice tumor cells were transduced with lentiviral vectors encoding shRNA for murine Stat3 or corresponding control vector as previously described (270). Briefly, the shRNA plasmids have been amplified in bacteria and transfected to HEK293 cells in combination with plasmids producing lentiviruses. The supernatant of the cells including viruses containing the plasmids was collected and transduced to the 4T1 cells. The plasmid clone with the most efficient Stat3 gene silencing (as evaluated on whole-cell lysates via western blotting) was selected for the 4T1 mouse model

iii. Western blot analysis *(paper III)*

Immunoblotting was performed in cultured cells and the procedure can be briefly described as follows: tumor cells were collected, washed in cold PBS and lysed in lysis buffer (including protease and phosphatase inhibitors) for protein extraction. Protein concentration was then

detected using a spectrophotometric microplate reader. Samples were prepared with the desired concentration for gel electrophoresis and electrotransfer using PVDF membrane. The membrane was subsequently blocked (milk in TBST) and incubated with the primary antibody of interest. Last, the secondary antibody was incubated and band detection was performed using chemiluminescence method. More details about the antibodies used can be found in paper III.

iv. RNA extraction, cDNA synthesis, and RT-qPCR *(paper III)*

Total RNA was extracted from cultured breast cancer cell lines using the RNeasy® Plus Mini Kit (QIAGEN Inc.) and cDNA was synthesized with Superscript First Strand Synthesis System (Invitrogen), both according to the respective protocols. The expression of mRNA was quantified by RT-qPCR using the Power SYBR® Green PCR Master Mix (ThermoFisher Scientific) and the comparative CT (ΔΔCt) method. 18S rRNA and beta-actin were used as the endogenous control genes. The RT-qPCR program (performed in a Veriti 96-well thermal cycler) included: i) Amplitaq Gold DNA polymerase activation (95 ◦C for 10'), ii) 40 cycles of DNA denaturation (95 ◦C for 15'') and iii) annealing (60 ◦C for 30'').

v. XTT proliferation assay *(paper III)*

4T1-shCTR and 4T1-shStat3 murine tumor cells were seeded for 3 days at a density of 4×10^3 cells/well (volume: 100ul each) in 96-well plates in complete RPMI-1640 medium. Six replicates were used for each cell line and time point (0, 24, 48 and 72 h). By using the Cell Proliferation Kit II, a mixture of XTT labeling with electron-coupling reagents was prepared per the manufacturer's protocol. For each time point, measurement of cell viability was performed using a spectrophotometer (wavelength:490nm; reference: 650 nm).

3.8 In vivo studies and animal models

i. 4T1 breast cancer animal model and evaluation of the tumor growth *(paper III)*

2×10^5 4T1 (shCTR and shStat3) cells in a volume of 50 mL PBS were injected into the mammary fat pad of anesthetized 4-6 week-old female BALB/c mice (Charles River Laboratory). Mice were sacrificed 3 weeks after injection and tumors were dissected and weighed. Tumor size was measured using calipers, and tumor volumes were estimated based on the following equation: $V = 4/3\pi \times (d/2)2 \times D/2$, where d is the minor tumor axis and D is

the major tumor axis. Ethical permits regarding *in vivo* model were obtained from the Swedish Board of Agriculture (N95/15).

ii. Flow Cytometric analysis of the immune microenvironment *(paper III)*

Mice tumors were dissected into smaller pieces using scalpels, minced in dissociation buffer (TrypLe and Stem Cell Pro Accutase (Life Technologies); 1:1) and incubated at 37 ◦C for 30' to the level of single-cell suspensions. Cell suspensions were subsequently passed through a 19 G syringe needle, filtered and washed in PBS (including 10% FBS). For the flow cytometry analysis and in order to prevent nonspecific antibody binding, single-cell suspensions were incubated with anti-CD16/32 monoclonal antibody (BioLegend) on ice 15' prior to the specific antibodies (for extracellular markers) incubation for 30' on ice. T-regulatory (FoxP3+) cells were also stained following manufacturer's instructions (BD Biosciences). Cells' viability was verified using 7AAD or the Live/Dead fixable dead cell stain (Life Technologies). Samples were proceeded in the BD Biosciences LSR II flow cytometer and analyzed in FlowJo software (Tree Star, Ashland). Further details regarding antibodies and methods can be found in the respective paper III.

iii. Pulmonary metastases colony assay *(paper III)*

Lungs from the sacrificed were dissociated to the single cell level using an enzymatic buffer (RPMI, 5% FBS, 0.2 mg/mL collagenase IV, 0.2 mg/mL dispase (Life technologies) and 0.1 mg/mL DNAse I (Sigma-Aldrich)) as described elsewhere (271-273). Cell suspensions were plated in the presence of 6-thioguanine (60uM) and the resistant ones were allowed to form colonies for 10 days and subsequently fixed with methanol, stained with crystal violet, and manually counted under a dissection microscope.

3.9 Systematic reviews and meta-analyses

i. Search strategy and study selection *(papers I and IV)*

A trial-level meta-analysis of studies reporting on the prognostic value of PD-L1 (paper I) and PD-1 (paper IV) protein expression was conducted. A systematic electronic search was performed by a librarian at the Karolinska Institutet University Library in November 2018 and updated in May 2019, for the PD-L1 and PD-1 meta-analyses, respectively in the following databases: Medline (Ovid), Embase, Cochrane Library (Wiley) and Web of Science Core

Collection. English language was set as a restriction. The full search strategies can be found in the Supplementary Methods of both papers I and IV. Manual reference reviewing of selected review articles on the topic and of potentially eligible articles was performed in order to ensure that all relevant studies were included: Only studies which fulfilled the following criteria were included in our meta-analysis: studies investigating the prognostic role (measured as time-to-event outcome) of PD-L1 expression (in tumor or immune cells) and PD-1 expression in patients with early-stage BC. We therefore excluded studies evaluating binary outcomes (as pathologic complete response) only, case reports, reviews, and meta-analyses. Two independent investigators performed study selection and consensus was reached in all eligible studies.

ii. Data extraction and quality assessment *(papers I and IV)*

Data were independently extracted by two investigators on a predefined form and a third investigator compared the databases for any discrepancies. The concordance rate between the 2 investigators was 97.1% for the PD-L1 (paper I) and 97.8% for the PD-1 (paper IV) meta-analyses. The data collected from each study included: first author's last name, publication year, country where the study was conducted, retrospective or prospective type of study; method of PD-L1/PD-1 evaluation, tissue used for analysis, threshold for PD-L1 expression, antibody used; positivity rate of PD-L1 expression in tumor cells and/or immune cells and PD-1 expression; study cohort characteristics of, follow-up time; outcome (time-to-event variables) for all patients data and within different BC subtypes -whenever feasible- and results from univariate and multivariable analyses

The methodological quality of each eligible study was evaluated by two independent investigators using the tumor biomarker prognostic studies 20-item REMARK checklist (274), generating a score (maximum 40). A third investigator resolved any discrepancies. The concordance rate between the 2 investigators regarding quality assessment was 74.2% for the PD-L1 (paper I) and 70.0% for the PD-1 (paper IV) meta-analyses.

3.10 Statistical Analyses

i. Statistical methods used in the analyses of original study cohorts

The chi-squared (χ^2) test and Fisher's exact tests were performed for the associations between categorical variables and assess any differences in clinicopathological characteristics between patient subgroups while Spearman's rank correlation coefficient was used to evaluate the

associations between continuous variables. Student's t-test and two-way ANOVA were used for comparisons between mean values on two groups

Survival outcomes in the relevant groups were estimated using Kaplan-Meier curves and compared with the log-rank test. The *survival* R package was used for such analyses using the indicated endpoints.

Univariate and multivariable Cox proportional hazards (PH) regression models were applied and unadjusted and adjusted HR and associated 95% CIs were estimated. The PH assumption was tested for all variables using the scaled Schoenfeld residuals. The set of covariates included the Cox models was selected on the basis of comparability and data availability among the training and validation cohorts as indicated and commonly included age, tumor size, lymph node status, subtypes and treatment. Exploratory interaction tests between the biomarker of interest and predefined clinical or PAM50-based subgroups and with survival outcome were evaluated in multivariable models.

Specifically in paper II, the added prognostic value of *PD-L1* gene to each gene-signature score was assessed using (a) the changes in the likelihood ratio test values (LR-$\Delta\chi^2$), and (b) the concordance index (c-index: measure of goodness of fit for binary outcomes in a logistic regression model). Each gene-signature score was subsequently added either alone, or in combination with *PD-L1* transcript expression to a Cox PH model with the corresponding clinical endpoint(s) for each cohort.

In particular in paper V, the area under the receiver operating characteristic (ROC) curve was used for the performance of the additional exploratory predictive models. Cohen's kappa coefficient was also used to measure agreement between molecular subtype assignments, as indicated whereas multiple testing in the GSEA was controlled by estimating the false discovery rate (FDR) (275).

A p-value less than 0.05 (two-tailed) was used and considered as statistically significant**.** All statistical analyses were done in R/ Bioconductor computing environment (versions 3.3.2, 3.5.1 and 3.6.2) and GraphPad Prism software version 7.0 (GraphPad Software Inc., San Diego, CA, USA) as indicated, unless otherwise stated.

ii. Statistical methods for trial-level meta-analyses

The first outcome of interest for the meta-analysis presented in paper I was the pooled PD-L1 positivity rate in tumor cells/immune cells, or both. A random effects model was used for the calculation of an overall pooled PD-L1 expression positivity rate and also the respective 95%

CI, only if there were at least 3 studies (the same in each subgroup). The different subgroups where positivity rate was calculated were distinguished i) per BC subtype based on IHC criteria (ER+/HER2-, HER2+ and TNBC), ii) per antibody used, iii) per type of tissue (TMA versus whole-tissue sections) and iv) according to the threshold of positivity used. In order to test for differences of pooled rates among subgroups, chi-square statistics were used. A case was considered as PD-L1 positive when the respective protein expression by IHC was greater than the threshold used in each specific eligible study.

The prognostic value of PD-L1 and PD-1 positivity was the second outcome of interest and time-to-event variables included DFS, RFS (end points merged in the meta-analysis given their almost identical definition) and OS. First, HR were calculated for all included studies for these specific variables as described in detail in papers I and IV. For each outcome of interest, HRs from both univariate and multivariate analyses for each eligible study were extracted and whenever possible meta-analyses were performed by pooling adjusted and unadjusted HRs separately. A threshold of 3 studies was set for subgroup analyses.

The presence of statistical heterogeneity among the studies was assessed using the Q statistics and the magnitude of heterogeneity using the I^2 statistic. A p-value <0.10 or an I^2 value>50% denoted substantial heterogeneity. If substantial heterogeneity was observed, the fixed-effects model was used, otherwise we used the random-effects model. Moreover, potential publication bias was evaluated qualitatively using a funnel plot. Statistical analyses were performed using RevMan 5.3 (Review Manager, Version 5.3) and StatsDirect (StatsDirect Ltd. UK, 2013) and significance threshold for all reported p values (2-sided) was $p < 0.05$.

iii. Statistical methods for pooled gene expression analysis

Associations between PD-L1/PD-1 mRNA expression levels and DFS/DMFS/RFS/PFI and OS were investigated. Univariate and multivariate Cox regression models with scaled expression values (continuous variables) and stratification by cohort were fitted, and unadjusted and adjusted HR and CI were estimated. The multivariate Cox regression model, was adjusted for age, tumor size, lymph node involvement, histological grade, ESR1 and ERBB2 expression status (not in models by molecular subtype). Each METABRIC study site (n=5) was treated as separate cohort. All data analysis was done in R/Bioconductor (version 3.5.2).

4 RESULTS AND DISCUSSION

Paper I: Prognostic implications of PD-L1 expression in breast cancer: systematic review and meta-analysis

Given that the clinical utility of PD-L1 expression in BC remained questionable and in light of prior inconclusive reports, we aimed to comprehensively investigate PD-L1 protein positivity rates and the prognostic value of this biomarker both at the protein and mRNA levels in early BC through the performance of i) a systematic review and trial-level IHC-based meta-analysis and ii) a pooled gene expression analysis of 39 publicly available transcriptomic datasets (see methods above). Pooled PD-L1 positivity in tumor cells, immune cells or both in the entire population, per subtype, per antibody and was examined as well as its prognostic value for DFS/RFS and OS.

The databases Medline, Embase, Cochrane Library and Web of Science Core Collection were searched up to November 2018 and data extraction was performed by two independent researchers. Of the initial 4,184 entries identified (2,746 entries upon de-duplication), 38 retrospective studies fulfilled the predefined inclusion criteria for the trial-level meta-analysis. The overall pooled PD-L1 positivity rate in tumor cells was 24% (95% CI, 15–33%; 20 studies; n=10,404), 33% (95% CI, 14–56%; 5 studies; n=4,696) in immune cells and 25% (95% CI, 3-59%; 4 studies; n=985) in both cell types. PD-L1 protein was mostly expressed in the triple-negative BC subtype. Regarding antibody performance, the highest tumor positivity rate was reported with Dako 28-8 clone (39%; 95% CI, 26-52%, p<0.001).

In the entire population, PD-L1 protein expression in tumor cells was an independent prognostic factor for shorter DFS (HR=1.62; 95% CI 1.14 – 2.33, p=0.008; I^2 = 64%, $p_{heterogeneity}$ =0.003) and OS (HR=1.93; 95% CI 1.20 – 3.09, p=0.006; I^2 = 80, $p_{heterogeneity}$ <0.001) as demonstrated in the multivariable analyses. In contrast, PD-L1 expression in immune cells was correlated with improved DFS (univariate HR=0.61; 95% CI 0.51 – 0.73, p<0.001; I^2 = 17%, $p_{heterogeneity}$ =0.29; 8 studies, n=969) and OS (univariate HR=0.53; 95% CI 0.39 – 0.73, p<0.001; I^2 = 0%, $p_{heterogeneity}$ =0.57; 7 studies, n=857) in TNBC, with no significant heterogeneity in these subgroup analyses.

In addition, higher PD-L1 gene expression was independently associated with improved survival in the entire population (multivariable HR=0.69, 95% CI 0.60 – 0.79, p<0.001, n=1,755 and HR=0.82, 95% CI 0.74 – 0.90, p<0.001, n=3,371 for DFS and OS, respectively) and in basal-like tumors (multivariable HR=0.53, 95% CI 0.38 – 0.74, p<0.001 and HR=0.64, 95% CI 0.52 – 0.80, p<0.001 for DFS and OS, respectively). Significant interaction was

between PD-L1 expression and molecular subtype was observed only in the OS analysis ($p_{interaction}$0.005)

PD-L1 expression as assessed by IHC is a clinically relevant biomarker in certain malignancies, since it may select appropriate candidates for immune checkpoint inhibition. The emerging role of PD-L1 expression and targeting especially in BC is reflected in the recent approval of the first anti-PD-L1 monoclonal antibody atezolizumab in combination with nab-paclitaxel in metastatic TNBC (203). Given that most of the benefit was demonstrated in the PD-L1 positive population and in light of several inconclusive reports using various IHC antibodies, scoring methods and cut-offs, there is an unmet need of discovering and validating predictive biomarkers.

This study represents the largest meta-analysis on PD-L1 IHC expression and informs on PD-L1 positivity rates and antibody selection as well as for the prognostic information of tumor versus immune cell expression. This is of interest for the design of clinical trials and sample size calculations since PD-L1 IHC expression selects a patient group with differential prognosis as candidates for immunotherapy.

On the other hand, PD-L1 gene expression seems to represent a promising prognostic factor predicting better outcomes, especially in basal-like BC, as demonstrated in pooled gene expression analyses including a large number of patients. This finding is line with the higher levels of PD-L1 protein in the TNBC which can be attributed in the higher genetic and transcriptomic heterogeneity and immunogenicity of this molecular subtype (33). Of note, the observed discrepancy in the prognostic information between protein and mRNA levels, might be related to PD-L1 gene expression in the stroma. Therefore, there is a need for methodological standardization (both for antibody validation and for transcript detection) before implementation in the clinical routine and its use as a biomarker of treatment selection. Of note, PD-L1 expression was higher when evaluated in whole tissue sections as compared to tissue microarrays, supporting the use whole sections whenever available.

Nonetheless, our study suffers from certain limitations that need to be addressed. The results among various studies are highly heterogeneous since different antibodies, cut-offs and scoring methods are used. The different patient populations and endpoint definition as derived from this trial-level (instead of individual patient level) meta-analysis also contribute to the substantial between-study heterogeneity, which was confirmed with the use of statistical tests. In addition, the limited number of studies did not allow for further subgroup analyses while the presence of publication bias might overestimate the true effect. Limitations and biases generated by pooled analyses of publicly available transcriptomic datasets have been previously described (276).

Paper II: PD-L1 mRNA expression as a prognostic marker in early BC patients and its additional prognostic value to 21-gene and 70-gene signature in ER+/HER2- disease

Having summarized and demonstrated the prognostic value of PD-L1 expression (paper I) and given the few published studies including IHC and RNA data in the same cohort we aimed to a) compare the prognostic implications of PD-L1 expression between at the protein and mRNA levels and b) investigate if PD-L1 gene expression could provide added prognostic value in validated gene signatures, especially in ER+/HER2- subtype.

The patient cohorts included i) our study cohort 1 (see Methods above, the "Merck" cohort) consisting of 562 primary BC patients with available gene expression profiling, tumor characteristics, treatment and follow-up data and ii) The Cancer Genome Atlas (TCGA) dataset consisting of 1081 patients with RNA-sequencing data (cohort 2).

Immunohistochemical staining was performed in TMAs for anti-PD-L1 (Ventana; clone SP263) antibody and membranous positivity was defined separately in tumor and in immune cells (i.e macrophages, dendritic cells). PD-L1 was evaluable in 490/562 (87.2%) cases and expressed in tumor cells, immune cells and any cell type in 9.8%, 23.7% and 24.7% of the patients respectively (see *Paper II: Figure 2*) and it was highly expressed among triple-negative/basal-like subtypes. PD-L1 protein expression both in the tumor and immune cells was significantly correlated with better DMFI (univariate HR=0.58; 95% CI=0.39-0.87, $p<0.01$ and multivariable HR=0.52; 95% CI=0.34-0.80; $p<0.01$) and OS (univariate HR=0.75; 95% CI=0.53-1.05, $p=0.089$ and multivariable HR=0.66; 95% CI=0.46-0.94; $p<0.05$). PD-L1 protein was statistically significantly correlated with gene expression levels (Wilcoxon Mann-Whitney, $p< 0.001$). Of note, tumors with higher PD-L1 transcript levels, were independently associated with better DMFI (HR=0.71; 95% CI=0.61-0.82; $p<0.001$) and OS (HR=0.77; 95% CI=0.67-0.87; $p<0.001$) both in the in the entire population as well as within basal-like and ER+/HER2- (HR=0.71; 95% CI=0.57-0.87; $p>0.01$) subtypes**.** Similar results were noted for PD-L1 gene expression with better PFI in the entire population in TCGA cohort (multivariable HR=0.76; 95% CI=0.64-0.91; $p<0.01$).

We also demonstrated that PD-L1 gene expression added significant prognostic information beyond that of 21-gene signature (LR-$\Delta\chi^2$=15.289 and LR-$\Delta\chi$2=8.812; $p<0.01$ for DMFI and PFI in cohorts 1 and 2, respectively) and the 70-gene signature (LR-$\Delta\chi$2=18.198 and LR-$\Delta\chi$2=8.467; $p<0.01$ for DMFI and PFI in cohorts 1 and 2, respectively) alone using the likelihood ratio approach. Similar improved results were demonstrated for the addition of PD-L1 gene to both 21-gene and 70-gene signature by using the c-indices method (see *Paper II:Table 2*) (277).

We also investigated the interplay among PD-L1 expression and immune cell subpopulations. Both PD-L1 protein and gene expression were correlated with increased IHC-determined CD3+ T-cell ($r=0.41$, $p<0.001$), expression in a subset of 412 patients. CD3 expression was higher in triple-negative/basal-like subtypes and was also associated with improved survival. Furthermore, PD-L1 gene expression was positively associated with CD8+ ($r=0.62$, $p<0.001$) and CD4+ memory activated ($r=0.66$, $p<0.001$) but not with memory resting ($r=-0.063$, $p=0.14$) T-cells or T-regs ($r=-0.12$, $p<0.01$) as evaluated *in silico* (see *Paper II: Supplementary Figure S4*).

Even though PD-L1 expression has been correlated with better prognostic capacity in early BC, the role of PD-L1 protein still remains inconclusive. To overcome the previous inconsistent results and analytical difficulties, we evaluated PD-L1 protein and gene expression at the same patient cohort. We showed in this study, that the use of this biomarker can improve prognosis of early BC patients. Therefore, while it seems that the prognostic information at the protein expression is more assay-dependent, PD-L1 gene expression might represent a promising factor. Moreover, a statistically significant correlation between protein and transcript levels was noted, as compared to previous reports (278, 279).

Given that the currently used gene-expression signatures are mainly proliferation and estrogen receptor signaling-based and do not consider immune response, there might be room for further refinement as prognostic and predictive tools. The role of immune infiltration in combination with prognostic scores (immunohistochemistry-based IHC4 test including ER, PR, HER2 and Ki67; CTS, clinical treatment score including node status, size, grade, age and treatment; ROR; RS) for predicting risk of recurrence after endocrine therapy has been implied in a recent study. (280) We demonstrated in this study that PD-L1 can provide significant additional prognostic value to the prospectively validated 21- and 70-gene signatures in patients with ER+/HER2- early BC. This finding -upon proper validation- can lead to improved prognostication in these patients where gene signatures are indicated.

In addition, in order to further delineate specific immune infiltration components in correlation with PD-L1, we immunohistochemically stained for the CD3 T-cell marker and we also analyzed the immune-related gene expression profiles using the bioinformatic tool CIBERSORT in the same patient cohort. We observed that PD-L1 expression associated with enhanced T-cell infiltration and therefore may represent a surrogate of effective anti-tumor immunity.

Whether the observed effect of PD-L1 can be solely attributed to the prognostic information or it is associated is chemotherapy prediction (see also paper V) remains questionable. The

potential predictive role of PD-L1 as a driver of chemosensitivity in the adjuvant setting of ER+/HER2- BC, could not be demonstrated here due to limited number of time-to-event and lack of randomization to chemo/no chemo-treated arms and therefore merits further investigation. Nevertheless, the clinical cohorts used in this study included both lymph node-positive and negative patients, thus hindering the direct clinical interpretation of our results on the added prognostic value to gene-signatures in this setting. Therefore, it is still unclear if it can identify patients with low risk of relapse who can safely forego adjuvant chemotherapy. Another limitation of this study is that the gene-signatures used are not the commercial version and the lower gene representation may affect their prognostic performance. Moreover, due to the retrospective nature of the included cohorts, we only managed to demonstrate the clinical validity of PD-L1 expression as a biomarker in early BC. In order to prove any possible clinical utility, prospective clinical trials needed to be designed. In addition, the evaluation of PD-L1 was performed on TMAs instead of whole tissue sections, the spatial heterogeneity of expression might be underestimated (281).

Paper III: STAT3-mediated the regulation of Programmed-Ligand 1 expression and immune profile modification in BC

Having previously reviewed the complex, multi-level regulation of PD-L1 (140), we aimed to explored potential mechanisms of PD-L1 regulation in BC, focusing on the oncogene/transcription factor STAT3. We showed that in breast cancer patients, a pSTAT3-GS was positively significantly associated with PD-L1 protein positivity (Wilcoxon, $p = 0.0027$, n=539) and PD-L1 gene expression in two independent patient cohorts (Spearman's rho = 0.34, $p<0.01$ and Spearman's rho = 0.38, $p < 0.01$ in the Merck cohort and TCGA, respectively). We also demonstrated in these two cohorts that pSTAT3 and PD-L1 transcripts were highly expressed in triple-negative as compared to non-TNBC subtypes.

In order to further confirm these findings, we evaluated protein expression in three different breast cancer cell lines using immunoblotting and immunohistochemical methods. We demonstrated that both pSTAT3 and PD-L1 presented with higher expression in TNBC cell lines (MDA-MB-231 and BT549) compared the non-TNBC cells (MCF7). Given these correlations and the lack of genetic amplification of PD-L1 in the cancer cell lines used (also shown in our study), we explored potential STAT3-mediated regulatory mechanisms of PD-L1. By using the selective pharmacologic STAT3 inhibitor C188-9 (XIII) and siRNA STAT3 gene knockdown we observed a downregulation of PD-L1 expression. In addition, overexpression of STAT3 gene expression using a constitutively actively plasmid as well as treatment with IL-6, led to increased pSTAT3 and PD-L1 expression. Taken together, we provided evidence that STAT3 transcriptionally regulated PD-L1 expression in breast *in vitro*. Furthermore, when Stat3 gene was silenced in the murine triple-negative 4T1 tumor cell line, PD-L1 gene was also down-regulated. In order to evaluate the effects of Stat3 gene silencing in vivo, we injected the mouse tumor cells (shCtrl and shStat3) into mammary fat pad of balb/c mice and generated a murine 4T1 breast carcinoma model. Stat3 gene silencing led to reduced tumor growth Day 25: 36.7%) and weight (Day 25: 31.3%) as well as to fewer pulmonary metastases compared to control mice. When investigating the immune infiltrate composition using flow cytometry, we demonstrated that Stat3 gene silencing was accompanied by anti-tumoral macrophage (increased F4/80+ and MHCII) and NK cell (CD69) accumulation but surprisingly by reduction of CD8+ T-cells and increase of T-regulatory cells, indicating the association of Stat3 with an altered immune profile.

Based on the observed macrophage phenotype shift *in vivo*, we also evaluated the expression pattern of macrophage phenotypes in breast cancer patients using immunofluorescence. We observed that pro-tumoral "M2-like" macrophage phenotype (CD163+) was correlated with

PD-L1 tumor cell protein expression and gene expression in breast tumors. Similar associations were noted among PD-L1 tumor cell expression and higher tumor grade and proliferation, thus indicating a biologically high-risk phenotype.

STAT3 represents an oncogene and an important transcription factor involved in several cellular functions, such as cell proliferation, invasiveness and metastasis (144) and recently in anti-tumor immune response, including immune checkpoints molecules and immune cell subpopulations in various tumor types (145). Even though the STAT3-mediated transcriptional regulation of PD-L1 has been previously reported (282, 283) a comprehensive approach on the role of STAT3 as a regulator of anti-tumor immune including *in vitro*, *in vivo* and patient material has not been reported in BC.

Upon Stat3 silencing, NK cell and macrophage accumulation as well as the skewness towards a more anti-tumoral "M1-like" phenotype significantly contributed to the reduced tumor growth and burden *in vivo.* The surprisingly observed CD8+ T-cell decrease and T-reg accumulation in the shSTAT3 tumors might be explained by the undetermined role of PD-L1 expression in immune cells (284) rather than only in tumor cells explored here. The decreased index of pulmonary metastases further underscores the impact of pro-tumoral "M2-like" in the metastatic potential (285).

We also showed that pro-tumoral macrophages were correlated to PD-L1 tumor cells expression in a subset of patients, contributing to an immunosuppressive phenotype. These finding are in line with the poor prognostic capacity of both PD-L1 tumor cell expression (paper I) and M2-like macrophages (100) in early breast cancer. Although few studies in other cancer types have reported such association (286) it is likely that the two distinct macrophage phenotypes can secrete cytokines and others factors which modifies PD-L1/PD-1 expression and anti-tumor immunity (287, 288).

The interplay of STAT3 with PD-L1 and immune profile in breast cancer might have potential clinical implications and pave the way for future therapeutic strategies with immune checkpoint blockade in combination with STAT3 inhibitors, since they are current ongoing trials testing their efficacy, especially in TNBC patients (289). Moreover, immune checkpoint inhibitors could be combined with macrophage accumulating and/or re-programming agents (e.g., CSF-1 inhibitors) in selected breast cancer patients, which are currently under clinical investigation (288, 290). Last but not least, identification of patient subgroups might lead to prognostic and predictive biomarker development.

Paper IV: Prognostic value of PD-1 protein and mRNA expression in early breast cancer

Despite the high interest on PD-1 blockade and the development of monoclonal antibodies, only few studies exist on the prognostic capacity of PD-1 expression in BC, which still remains unclear. Our study comprised three different parts: i) Immunohistochemical evaluation PD-1 protein on tissue microarrays and gene expression in our retrospective original study cohort comprising of 564 patients treated for early BC between 1997-2005 in Stockholm, Sweden (see also *methods: patient populations*); ii) Systematic review and an IHC study-based meta-analysis with literature search up to May 2019; and iii) Pooled analysis of transcriptomic data from 39 publicly available datasets (see also paper I).

In the study cohort, 220/564 (46.7%) of the patients demonstrated PD-1 positive protein expression in immune cells which was significantly correlated to characteristics such as estrogen receptor-negativity and high proliferation rate ($p<0.001$). Nevertheless, BC patients with high PD-1 protein expression were associated with improved DRFI (multivariable HR = 0.66, 95% CI 0.48 – 0.91, p=0.010) and OS (multivariable HR=0.73, 95% CI 0.55 – 0.97, p=0.027). In contrast, PD-1 gene expression was not found to significantly predict survival outcomes DRFI (HR=0.88, 95% CI 0.66 – 1.18, p=0.399) and OS (HR=0.88, 95% CI 0.68 – 1.13, p=0.312) as revealed in multivariable analyses. Moreover, no significant interaction was noted between PD-1 expression and IHC-based subtype in the subgroup survival analyses. A weak statistically significant correlation was also demonstrated between PD-1 mRNA and protein expression levels cohort (Spearman's rho=0.123, p=0.007).

In the first -to date- reported meta-analysis on the prognostic information of PD-1 expression, 4736 initial entries (3298 entries upon de-duplication) were identified. Study selection, data extraction (concordance rate = 97.8%) and quality assessment (concordance rate = 70%) were performed by two independent investigators and a total of 15 retrospective studies -including our study cohort- that fulfilled the predefined eligibility criteria were included in the analysis. PD-1 protein expression did not significantly predict survival outcomes in the entire population. Interestingly, it was correlated with improved DFS in the TNBC subtype (3 studies, n=417; pooled multivariable HR = 0.57; 95% CI 0.29 – 0.90; p-value = 0.02).

Furthermore, a pooled gene expression analysis from 39 available public databases (n= 9493) (see also paper I), including GEP data from our original cohort, was performed. High PD-1 gene expression was associated with significantly improved OS (pooled multivariable HR=0.89; 95% CI 0.80 – 0.99; p=0.025) but not DFS in the entire population. Within molecular subtypes, higher PD-1 transcript levels predicted better OS in the basal-like (pooled multivariable HR=0.77, 95% CI 0.63 – 0.95, p=0.014) and HER2-enriched (pooled

multivariable HR=0.78, 95% CI 0.61 – 0.98, p=0.032) subtypes, although no significant interaction was detected ($p_{interaction}$ = 0.185).

We demonstrated that PD-1 protein and gene expression correlated to improved prognosis in early BC patients, especially in the triple-negative/basal-like subtypes, using a multi-level approach (the only to date meta-analysis on PD-1 IHC expression and the largest pooled gene expression analysis). Similar prognostic capacity in these highly immunogenic subtypes was also noted for PD-L1 expression (see paper I) -especially in the immune cells- further highlighting the crucial role of TME. Given that PD-1 is generally considered as a co-inhibitory immune molecule, a number of reports indicate that PD-1 could represent a marker of T-cell activation and receptor signaling rather than a T-cell exhaustion indicator (80, 130), which can be defined by other immune checkpoints (i.e TIM-3, LAG-3, CD39) (291), Of note, PD-1+CD8+ cells have been correlated to better survival (187), underscoring the need of a comprehensive understanding of the specific immune composition and expression patterns. PD-1 has been also linked to T-cell activation-associated gene expression in patients with BC (292), further indicating a marker of an effective anti-tumor immune response.

Of note, in terms of prognostic information in our study cohort, a discrepancy was noted between PD-1 protein and gene expression levels. PD-1 IHC analytical difficulties seem to be fewer, as compared to PD-L1 (178), since an antibody clone is commonly used and its expression is observed on immune cells (128). Nonetheless, the weak significant correlation observed between PD-1 mRNA and protein expression in the study cohort question the reliability of IHC evaluation of this marker, although data on PD-1 mRNA expression as a prognostic biomarker are scarce (187). Given the complex post-transcriptional/translational regulation of PD-1 expression (293), there is a need for further validation of the genomic detection methods and prospective evaluation of its clinical utility.

The limitations of this study include: i) the few retrospective studies which did not allow for further subgroup analyses, ii) the heterogeneous patient populations and IHC scoring methods which introduced significant heterogeneity, iii) the trial-level meta-analysis instead of an individual patient data analysis, iv) the evaluation of PD-1 protein on TMA rather than in whole tissue section which possibly underestimated the spatial expression heterogeneity in our study cohort which was also v) of a small size and vi) had an overrepresentation of biologically aggressive tumors (higher histological grade, proliferation rate, larger size, more lymph node metastases and younger patients); vi) biases and limitations introduced by the analysis on publicly available transcriptomic datasets.

Paper V: Immune gene expression and chemotherapy response in advanced BC

Considering the promising capacity of gene expression signatures as predictors of response to neoadjuvant chemotherapy in breast cancer, we investigated the predictive role of intrinsic molecular subtypes and modules including immune response, ER signaling, proliferation, PIK3CA and P53 mutations, using the gene expression profiles obtained at baseline from fine FNAB of metastatic BC tissue in 109 patients treated with first-line chemotherapy in the TEX phase III clinical trial (epirubicin and paclitaxel +/- capecitabine).

Two non-overlapping immune response signatures (48, 121) were used and one of them was statistically significantly correlated with higher probability of achieving objective response to chemotherapy (per standard deviation odds ratio (OR) = 1.62; 95% CI, 1.03–2.64; P=0.04) in the entire population. However, both investigated immune modules showed a consistently significant high predictive value in ER positive (per s.d. odds ratio (OR) 2.05, 95% CI 1.11 – 4.13, p=0.02 and per s.d OR 2.23, 95% CI 1.21 – 4.48, p=0.01 for the two modules) and luminal cancers (per s.d. odds ratio (OR) 6.91, 95% CI 2.11 – 35.05, p=0.006 and per s.d OR 3.54, 95% CI 1.43 –10.86, p=0.01), but not in ER-negative or non-luminal tumors. An independent GSEA confirmed that that immune-related gene sets (including PD-1 signaling and interferon-γ) were enriched in ER-positive or molecularly luminal responders.

In order to evaluate the extent of lymphocytic infiltration in this setting we performed H&E or Giemsa staining on FNAB material from various metastatic sites (evaluable smears on 50/109 patients) in order to generate a scoring algorithm for lymphocytes enumeration. We reported scarce TIL counts (median 1.3% of the total cells; IQR: 0.6-2.3%) and low correlation with immune modules (Spearman's rho= 0.26 and 0.18), with no predictive effect to chemotherapy. Moreover, for the *in silico* calculation of absolute abundance of immune and stromal cell types, we used the MCP-counter method. Lymphocytes and monocytes were associated with increased chemosensitivity in the ER-positive and luminal subgroups without outperforming the one observed with the two immune-related gene modules.

The favorable prognostic capacity of tumor-infiltrating lymphocytes has been demonstrated in triple-negative and HER2-positive BC patients both in adjuvant and neoadjuvant settings (39, 43). TILs also carry predictive information for benefit to neoadjuvant chemotherapy, leading to improved pCR rates (43), although not well studied in the metastatic setting. Moreover, even though gene expression signatures have been used for treatment prediction in the neoadjuvant setting (122), their predictive value in advanced breast cancer is largely unknown. Subgroup analyses of two large randomized immune checkpoint blockade clinical trials, IMPassion130

(163, 202) and KEYNOTE-119 (164), evaluating PD-L1 protein expression, revealed that the effect on PFS or OS respectively in the chemotherapy-only arm was the same irrespective PD-L1 status in patients with metastatic TNBC.

Taken together, we have shown for the first time that in patients with ER+ and luminal advanced BC, immune-related gene signatures could statistically significantly predict response to chemotherapy, paving the way for the development of novel immune-based predictive factors of chemosensitivity in luminal tumors which are considered as less immunogenic and highly heterogeneous (294). The results of this study indicate that an active immune microenvironment (as reflected by the use of largely non-overlapping immune signatures used in this study) may carry the predictive information and not specific genes, since a single marker is possibly unable to grasp the complexity of tumor-host interactions. Indeed, the release of neoantigens which can trigger anti-tumor immune response may be a crucial step towards the induction of immunogenic cancer cell death (198).

Furthermore, we tried to explore the predictive role of PD-L1/PD-1 a driver of chemosensitivity in the adjuvant setting of ER+/HER2- BC using both our study cohort as well as public available datasets (see paper II). This hypothesis could not be proven due to limited number of time-to-event and therefore merits further investigation in trials included chemotherapy-treated vs non-chemotherapy BC patients. To determine the predictive role of tumor immune infiltration for the effect of adjuvant chemotherapy especially in luminal BC, a large prospective trial is needed including both chemo-treated and untreated patients.

Nevertheless, there are limitations of our study which need to be acknowledged. There is no other similar metastatic cohort -to date- with available GEP data in order to validate our findings and investigate if the results can apply to other chemotherapeutic drugs or other treatment lines or treatment types. This concept needs to be further validated in a prospective randomized trial, considering the limitations emerged by the prospective-retrospective approach of this analysis and the small number of patients.

Although our hypothesis is that patients with high TILs are expected to better respond to chemotherapy, this could not be tested here since the numbers of lymphocytes detected were too low. Moreover, given the lack of TILs evaluation guidelines in the metastatic BC setting and the paucity of studies with available material from the various metastatic sites hinder the further validation our scoring method while number of TILs is also lower in metastases compared to the primary tumor (60). Inter-observer and intra-observer variability despite guidelines for the interpretation of slides, dependency on biopsy methodology and risk of sampling biases are also among the relative disadvantages of TILs as a biomarker in advanced

breast cancer. At the same time, fine needle aspiration biopsy material does not take into account the TIME interactions as compared to metastatic core biopsies and this could probably explain the lack of prediction in the ER-negative subgroup where the presence of stroma drives immunogenicity. However, despite the sampling difficulties, tumor heterogeneity in metastatic biopsies (295, 296) dictates a shift from the cancer cell-centric concept to that of the TME interactions and therefore a shift from a static to a dynamic, longitudinal evaluation of biomarkers.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Given the complex regulation, the emerging role of PD-L1/PD-1 signaling axis as a biomarker and immunotherapeutic target in BC and the complex interactions among tumor and immune cells, the present doctoral thesis aimed to shed light in some of these aspects. The conclusions and clinical implications that can be drawn from these studies are:

- PD-L1 protein expression in tumor cells is poor prognostic factor, whereas PD-L1 expression in immune cells is a favorable one in the TNBC subtype
- PD-1 protein expression is correlated with improved outcomes in basal-like/TNBC subtypes
- PD-1 and PD-L1 gene expressions are promising prognostic biomarkers, predicting improved survival outcomes especially in in basal-like/triple-negative subtypes
- PD-L1 mRNA expression adds significant prognostic value to prospectively validated 21- gene and 70-gene signatures in ER+/HER2- disease
- Immune-related gene signatures are predictive for chemosensitivity in luminal advanced breast cancer
- T-cell infiltration and pro-tumoral macrophages are correlated with PD-L1 protein expression
- STAT3 represents a regulator of PD-L1 expression in BC *in vitro* and modulates antitumor immune response mainly through macrophage phenotype shift, acummulation of NK cells and T-lymphocytes *in vivo*

External validation of our findings and standardization of PD-L1/PD-1 detection methods are of utmost importance. Protein evaluation in the immune cells and gene expression as biomarkers could improve prognostication and further refine currently available gene expression signatures and patient management algorithms. The main concept of immunotherapy with the release of immunological “brakes” which dampen the immune response through exhaustion cytotoxic T-lymphocytes has led to the introduction of immune checkpoint inhibitors. Even though PD-1/PD-L1 axis blockade has met with success during the past years with durable responses and increased efficacy, there are still two major barriers that need to be considered before for the integration of such new therapies: toxicity and financial cost. Close monitoring of the patients will contribute to the recognition of the immune-related adverse events and enhance the potential for combinational immune checkpoint inhibition. At the same time, immunotherapy revolution is accompanied by increased cost and thus cost-effectiveness issues should be also considered for each treatment option. Moreover, despite the

exciting developments in this rapidly evolving field, only a minority of the potential immune-related markers and therapeutic targets have been comprehensively investigated. Other determinants of anti-tumor immune response need to further clarified using the emerging multiplexed imaging and multi-omics technologies, which might expand the therapeutic armamentarium for BC patients. More research is also warranted towards further elucidation of the underlying regulatory mechanisms driving PD-L1/PD-1 expression and antitumor immunity as well as their clinical implications especially in the highly heterogenous BC in order to enter the new era of personalized cancer medicine.

5 ACKNOWLEDGEMENTS

I would like to express my gratitude to Karolinska Institutet (KI) for providing me a high-class research environment, the opportunity to interact with various talented researchers and a basis of new ideas in order to conduct translational cancer research.

During this exciting journey towards accomplishment of a PhD, I would like to thank a lot of people who supported and accompanied me towards the completion of my doctoral studies:

My principal supervisor **Associate Professor Theodoros Foukakis** for many many reasons. First of all, you believed in me and gave me the opportunity to follow the PhD path into cancer research, that I was dreaming of. You have been an excellent and generous supervisor, you encouraged me through all this way and shaped an ideal environment for me to work in. You have always been an inspiration to me and I hope that I fulfilled your expectations. Your trust to me as well as the room for initiatives and interaction in multiple levels has led to my scientific and personal development. I have learnt so many things from your expertise and your valuable guidance. Your kindness and optimism as a person have also contributed to the generation of a great team spirit and I appreciate your patience, while listening to all my ideas. I am really grateful for all these opportunities and I am looking forward to our next steps! Thank you!

Professor Jonas Bergh for your continuous support throughout my time in your group. Your expertise, crucial input and scientific guidance through evidence-based discussions motivated me in order to deepen my knowledge into the field breast cancer and oncology. I sincerely appreciate all the nice working environment that you have provided, lab meetings and purposeful comments. Thank you for all your trust and advice and for being an active member in the group.

Professor George Rassidakis for your guidance and encouragement during my PhD studies. You introduced me in the principles of laboratory cancer research and pathology from my very first steps. Thank you for all your patience and time during the evaluation of breast cancer tissue slides and research results. Our scientific discussions really sharpened my thinking about cancer biology and tissue morphology. You have closely followed my development and I really appreciate all your support in various levels.

Associate Professor Charlotte Rolny for your help and support all these years. Your scientific input broadened my knowledge and interest in tumor immunology, cancer biology and preclinical studies. Your research group has always been always helpful to me and thank you for our fruitful collaboration.

Alexis Matikas for being an excellent collaborator and a true friend. From being the first person to introduce me in an Oncology Clinic to our recent times in Karolinska, you have always been helpful, supportive and a source of motivation to me. I really appreciate your willingness to share your knowledge, listen to my ideas, answer my questions and guide me throughout our projects and thank you for all the productive and nice moments throughout these years.

Susanne Agartz for accompanying me during everyday lab work, your kindness, effective collaboration, ability to solve various issues, personal discussions and all nice moments. Thank you for your patience on answering my "one question per day"!

Andri Papakonstantinou for all the nice moments and encouragement. From being one of the first persons that I met in Karolinska up to our "neighboring" dissertations, you have always been supportive and kind to me. I appreciate your advice, enthusiasm, organizing skills (both for the courses and for the thesis!) and all your help during my PhD journey.

Helen Eriksson for your assistance and willingness to help with any issue arising. Thank you for your kindness!

Emmanouil Sifakis for your guidance, nice collaboration and your valuable input in our projects. **Nick Tobin** for all you help, interesting discussions and for serving as a chairman in my dissertation. **John Lövrot** for our collaboration and assistance. Thank you all for introducing me into basic principles of biostatistics and bioinformatics through our multidisciplinary meetings.

Associate Professor Antonis Valachis, Professor Christos Sotiriou and **Dr François Richard** for the fruitful collaboration, expertise and scientific input throughout our projects.

Associate Professor Thomas Hatschek for providing valuable patient material and for your kind attitude and help and **Professor emeritus Lambert Skoog** for his assistance in the evaluation of FNAB.

Amandine Anastacio, Xia Chao, Associate Professor Kenny Rodriguez-Wallberg, Markella Zacharouli, Aafke Duijnmejer, Johanna Klinge, **Arian Lundberg, Per Rydberg** and **Anestis Sofiadis** for the help, nice atmosphere and talks.

Maijken Wallerius, Tatjana Wallman, Margarita Bartish, Christos Coucouravas and **Jing Jing Ye** for your valuable help with *in vivo* and *in vitro* studies, your friendly attitude and the nice discussions we had together. **Georgia Kokaraki** for your help with patient material and IHC stainings.

Associate Professor Johan Hartman for your assistance in digital pathology and the nice collaboration with your research group: **Lisa Viberg, Xinsong Chen, Ran Ma** and **Balazs Ács.**

Artur Mezheyeuski for the excellent collaboration. I am looking forward to our upcoming research projects.

Dimitris Salgkamis for the nice work and your friendship.

Associate Professor Andreas Lundqvist and **Erika Rindsjö** for all the help and collaboration and nice interaction in the Doctoral Education Committee in the Department of Oncology-Pathology.

Professor George Pentheroudakis from University of Ioannina for being my mentor and your overall guidance in my early steps. Thank you for your advice and inspiration towards pursuing a career in Medical Oncology.

My dear friends: **Giannis Mantas** for all the special moments that we have experienced. You have always been there for me and helped me in various ways. You are a source of motivation and our discussions developed my way of thinking. **Nikos Tsezmetzis** for almost being my toast master, for being my supervisor in the lab during my first steps in Karolinska and for being an excellent friend to me, sharing your sense of humor and positive vibes. **Sebastian Chrétien** for all the nice moments during your stay in Stockholm. I am so glad that I met you and I hope to your return. **Maria Kalomoiri** for being a true friend and for all your support!

Muyi for your kind friendship and all the late-night shifts in the lab. **Pablo, Sander, Christina, Robert, Adam, Marcus, Konstantina, Francesca, Michele, Pedro, Mathilde, Patricia** and **Vassilis** for all your help the nice moments together.

Ioanna Markaki for all your multilevel support, useful advice and beautiful moments together.

Maria Kouvaraki for your collaboration, advice, kindness and generous hospitality.

Kostas Arvanitakis, Elpida Kouimtzi, Eirini Makrygiannaki, Chrysa Giannakaki, **Thodoris Velentzas** and **Giorgos Georgountzos** for your generous friendship and support all these years!

My young friends: Oliver, Electra, Ioanna, Athina, Ianthe and **Michaela** for all your smiles and playful happy moments.

My whole **family** for all their love, encouragement and support all these years.

Μπαμπά, Μαμά, Γεωργία σας ευχαριστώ πολύ για τη στήριξη, την εμπιστοσύνη και την αγάπη σας όλα αυτά τα χρόνια!

To my **Lilly** for your endless support, unconditional love and for being always on my side. Thank you for your patience and care during all these years. Hope that I can give you back a part of all you have done for me.

6 REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018;68(6):394-424.

2. Berry DA, Cronin KA, Plevritis SK, Fryback DG, Clarke L, Zelen M, et al. Effect of screening and adjuvant therapy on mortality from breast cancer. The New England journal of medicine. 2005;353(17):1784-92.

3. Ries L, Melbert D, Krapcho M, Mariotto A, Miller B, Feuer E, et al. SEER Cancer Statistics Review, 1975-2004, National Cancer Institute. Bethesda, MD. 2007.

4. Verdecchia A, Francisci S, Brenner H, Gatta G, Micheli A, Mangone L, et al. Recent cancer survival in Europe: a 2000-02 period analysis of EUROCARE-4 data. The Lancet Oncology. 2007;8(9):784-96.

5. Foukakis T, Fornander T, Lekberg T, Hellborg H, Adolfsson J, Bergh J. Age-specific trends of survival in metastatic breast cancer: 26 years longitudinal data from a population-based cancer registry in Stockholm, Sweden. Breast cancer research and treatment. 2011;130(2):553-60.

6. Pan H, Gray R, Braybrooke J, Davies C, Taylor C, McGale P, et al. 20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years. The New England journal of medicine. 2017;377(19):1836-46.

7. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science (New York, NY). 1987;235(4785):177-82.

8. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2011;24(2):157-67.

9. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.

10. Peto R, Davies C, Godwin J, Gray R, Pan HC, Clarke M, et al. Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. Lancet (London, England). 2012;379(9814):432-44.

11. Increasing the dose intensity of chemotherapy by more frequent administration or sequential scheduling: a patient-level meta-analysis of 37 298 women with early breast cancer in 26 randomised trials. Lancet (London, England). 2019;393(10179):1440-52.

12. Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT, et al. Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-updagger. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(8):1194-220.

13. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. Lancet (London, England). 2014;384(9938):164-72.

14. Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials. The Lancet Oncology. 2018;19(1):27-39.

15. Ballman KV. Biomarker: Predictive or Prognostic? Journal of Clinical Oncology. 2015;33(33):3968-71.

16. Febbo PG, Ladanyi M, Aldape KD, De Marzo AM, Hammond ME, Hayes DF, et al. NCCN Task Force report: Evaluating the clinical utility of tumor markers in oncology. Journal of the National Comprehensive Cancer Network : JNCCN. 2011;9 Suppl 5:S1-32; quiz S3.

17. Hayes DF, Trock B, Harris AL. Assessing the clinical impact of prognostic factors: when is "statistically significant" clinically useful? Breast cancer research and treatment. 1998;52(1-3):305-19.

18. Foukakis T, Bergh J. Prognostic and predictive factors in early, non-metastatic breast cancer. Dizon Ed UpToDate. 2016.

19. Nicolini A, Ferrari P, Duffy MJ. Prognostic and predictive biomarkers in breast cancer: Past, present and future. Seminars in cancer biology. 2018;52(Pt 1):56-73.

20. Kwa M, Makris A, Esteva FJ. Clinical utility of gene-expression signatures in early stage breast cancer. Nature reviews Clinical oncology. 2017;14(10):595-610.

21. Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. The New England journal of medicine. 2009;360(8):790-800.

22. Sestak I, Buus R, Cuzick J, Dubsky P, Kronenwett R, Denkert C, et al. Comparison of the Performance of 6 Prognostic Signatures for Estrogen Receptor-Positive Breast Cancer: A Secondary Analysis of a Randomized Clinical Trial. JAMA oncology. 2018;4(4):545-53.

23. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Prospective Validation of a 21-Gene Expression Assay in Breast Cancer. The New England journal of medicine. 2015;373(21):2005-14.

24. Cardoso F, van't Veer LJ, Bogaerts J, Slaets L, Viale G, Delaloge S, et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. The New England journal of medicine. 2016;375(8):717-29.

25. Albain KS, Barlow WE, Shak S, Hortobagyi GN, Livingston RB, Yeh IT, et al. Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. The Lancet Oncology. 2010;11(1):55-65.

26. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Adjuvant Chemotherapy Guided by a 21-Gene Expression Assay in Breast Cancer. The New England journal of medicine. 2018;379(2):111-21.

27. Matikas A, Foukakis T, Swain S, Bergh J. Avoiding over- and undertreatment in patients with resected node-positive breast cancer with the use of gene expression signatures: are we there yet? Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(7):1044-50.

28. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

29. Finn OJ. Cancer immunology. The New England journal of medicine. 2008;358(25):2704-15.

30. Lanitis E, Dangaj D, Irving M, Coukos G. Mechanisms regulating T-cell infiltration and activity in solid tumors. Annals of oncology : official journal of the European Society for Medical Oncology. 2017;28(suppl_12):xii18-xii32.

31. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1-10.

32. Gruosso T, Gigoux M, Manem VSK, Bertos N, Zuo D, Perlitch I, et al. Spatially distinct tumor immune microenvironments stratify triple-negative breast cancers. The Journal of clinical investigation. 2019;129(4):1785-800.

33. Bareche Y, Buisseret L, Gruosso T, Girard E, Venet D, Dupont F, et al. Unraveling triple-negative breast cancer tumor microenvironment heterogeneity: towards an optimized treatment approach. Journal of the National Cancer Institute. 2019.

34. Karn T, Jiang T, Hatzis C, Sanger N, El-Balat A, Rody A, et al. Association Between Genomic Metrics and Immune Infiltration in Triple-Negative Breast Cancer. JAMA oncology. 2017;3(12):1707-11.

35. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature. 2013;500(7463):415-21.

36. Yarchoan M, Hopkins A, Jaffee EM. Tumor Mutational Burden and Response Rate to PD-1 Inhibition. The New England journal of medicine. 2017;377(25):2500-1.

37. Stanton SE, Adams S, Disis ML. Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes: A Systematic Review. JAMA oncology. 2016;2(10):1354-60.

38. Solinas C, Carbognin L, De Silva P, Criscitiello C, Lambertini M. Tumor-infiltrating lymphocytes in breast cancer according to tumor subtype: Current state of the art. Breast (Edinburgh, Scotland). 2017;35:142-50.

39. Loi S, Drubay D, Adams S, Pruneri G, Francis PA, Lacroix-Triki M, et al. Tumor-Infiltrating Lymphocytes and Prognosis: A Pooled Individual Patient Analysis of Early-Stage Triple-Negative Breast Cancers. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2019;37(7):559-69.

40. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2013;31(7):860-7.

41. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Annals of oncology : official journal of the European Society for Medical Oncology. 2014;25(8):1544-50.

42. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014;32(27):2959-66.

43. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. The Lancet Oncology. 2018;19(1):40-50.

44. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, et al. Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nature reviews Clinical oncology. 2016;13(4):228-41.

45. Park JH, Jonas SF, Bataillon G, Criscitiello C, Salgado R, Loi S, et al. Prognostic value of tumor-infiltrating lymphocytes in patients with early-stage triple-negative breast cancers (TNBC) who did not receive adjuvant chemotherapy. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(12):1941-9.

46. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Annals of oncology : official journal of the European Society for Medical Oncology. 2015;26(2):259-71.

47. Hoon Tan P, Ellis I, Allison K, Brogi E, Fox SB, Lakhani S, et al. The 2019 WHO classification of tumours of the breast. Histopathology. 2020.

48. Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(9):983-91.

49. Perez EA, Ballman KV, Tenner KS, Thompson EA, Badve SS, Bailey H, et al. Association of Stromal Tumor-Infiltrating Lymphocytes With Recurrence-Free Survival in the N9831 Adjuvant Trial in Patients With Early-Stage HER2-Positive Breast Cancer. JAMA oncology. 2016;2(1):56-64.

50. Kim RS, Song N, Gavin PG, Salgado R, Bandos H, Kos Z, et al. NRG Oncology/NSABP B-31: Stromal tumor infiltrating lymphocytes (sTILs) and outcomes in early-stage HER2-positive breast cancer (BC). American Society of Clinical Oncology; 2018.

51. Dieci MV, Conte P, Bisagni G, Brandes AA, Frassoldati A, Cavanna L, et al. Association of tumor-infiltrating lymphocytes with distant disease-free survival in the ShortHER randomized adjuvant trial for patients with early HER2+ breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(3):418-23.

52. Solinas C, Ceppi M, Lambertini M, Scartozzi M, Buisseret L, Garaud S, et al. Tumor-infiltrating lymphocytes in patients with HER2-positive breast cancer treated with neoadjuvant chemotherapy plus trastuzumab, lapatinib or their combination: A meta-analysis of randomized controlled trials. Cancer treatment reviews. 2017;57:8-15.

53. Dieci MV, Prat A, Tagliafico E, Pare L, Ficarra G, Bisagni G, et al. Integrated evaluation of PAM50 subtypes and immune modulation of pCR in HER2-positive breast cancer patients treated with chemotherapy and HER2-targeted agents in the CherLOB trial. Annals of oncology : official journal of the European Society for Medical Oncology. 2016;27(10):1867-73.

54. Salgado R, Denkert C, Campbell C, Savas P, Nuciforo P, Aura C, et al. Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and

Trastuzumab: A Secondary Analysis of the NeoALTTO Trial. JAMA oncology. 2015;1(4):448-54.

55. Ingold Heppner B, Untch M, Denkert C, Pfitzner BM, Lederer B, Schmitt W, et al. Tumor-Infiltrating Lymphocytes: A Predictive and Prognostic Biomarker in Neoadjuvant-Treated HER2-Positive Breast Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016;22(23):5747-54.

56. Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, et al. Assessing Tumor-infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method From the International Immunooncology Biomarkers Working Group: Part 1: Assessing the Host Immune Response, TILs in Invasive Breast Carcinoma and Ductal Carcinoma In Situ, Metastatic Tumor Deposits and Areas for Further Research. Advances in anatomic pathology. 2017;24(5):235-51.

57. Foukakis T, Lovrot J, Matikas A, Zerdes I, Lorent J, Tobin N, et al. Immune gene expression and response to chemotherapy in advanced breast cancer. British journal of cancer. 2018;118(4):480-8.

58. Luen SJ, Salgado R, Fox S, Savas P, Eng-Wong J, Clark E, et al. Tumour-infiltrating lymphocytes in advanced HER2-positive breast cancer treated with pertuzumab or placebo in addition to trastuzumab and docetaxel: a retrospective analysis of the CLEOPATRA study. The Lancet Oncology. 2017;18(1):52-62.

59. Liu S, Chen B, Burugu S, Leung S, Gao D, Virk S, et al. Role of Cytotoxic Tumor-Infiltrating Lymphocytes in Predicting Outcomes in Metastatic HER2-Positive Breast Cancer: A Secondary Analysis of a Randomized Clinical Trial. JAMA oncology. 2017;3(11):e172085.

60. Ogiya R, Niikura N, Kumaki N, Bianchini G, Kitano S, Iwamoto T, et al. Comparison of tumor-infiltrating lymphocytes between primary and metastatic tumors in breast cancer patients. Cancer science. 2016;107(12):1730-5.

61. Cimino-Mathews A, Thompson E, Taube JM, Ye X, Lu Y, Meeker A, et al. PD-L1 (B7-H1) expression and the immune tumor microenvironment in primary and metastatic breast carcinomas. Human pathology. 2016;47(1):52-63.

62. Dieci MV, Tsvetkova V, Orvieto E, Piacentini F, Ficarra G, Griguolo G, et al. Immune characterization of breast cancer metastases: prognostic implications. Breast cancer research : BCR. 2018;20(1):62.

63. Szekely B, Bossuyt V, Li X, Wali VB, Patwardhan GA, Frederick C, et al. Immunological differences between primary and metastatic breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2018;29(11):2232-9.

64. Duchnowska R, Peksa R, Radecka B, Mandat T, Trojanowski T, Jarosz B, et al. Immune response in breast cancer brain metastases and their microenvironment: the role of the PD-1/PD-L axis. Breast cancer research : BCR. 2016;18(1):43.

65. Hamy AS, Bonsang-Kitzis H, De Croze D, Laas E, Darrigues L, Topciu L, et al. Interaction between Molecular Subtypes and Stromal Immune Infiltration before and after Treatment in Breast Cancer Patients Treated with Neoadjuvant Chemotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2019;25(22):6731-41.

66. Li X, Warren S, Pelekanou V, Wali V, Cesano A, Liu M, et al. Immune profiling of pre- and post-treatment breast cancer tissues from the SWOG S0800 neoadjuvant trial. Journal for immunotherapy of cancer. 2019;7(1):88.

67. Waks AG, Stover DG, Guerriero JL, Dillon D, Barry WT, Gjini E, et al. The Immune Microenvironment in Hormone Receptor-Positive Breast Cancer Before and After Preoperative Chemotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2019;25(15):4644-55.

68. Garcia-Martinez E, Gil GL, Benito AC, Gonzalez-Billalabeitia E, Conesa MA, Garcia Garcia T, et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. Breast cancer research : BCR. 2014;16(6):488.

69. Luen SJ, Salgado R, Dieci MV, Vingiani A, Curigliano G, Gould RE, et al. Prognostic implications of residual disease tumor-infiltrating lymphocytes and residual cancer burden in triple-negative breast cancer patients after neoadjuvant chemotherapy. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(2):236-42.

70. Dieci MV, Criscitiello C, Goubar A, Viale G, Conte P, Guarneri V, et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: a retrospective multicenter study. Annals of oncology : official journal of the European Society for Medical Oncology. 2014;25(3):611-8.

71. Miyashita M, Sasano H, Tamaki K, Hirakawa H, Takahashi Y, Nakagawa S, et al. Prognostic significance of tumor-infiltrating CD8+ and FOXP3+ lymphocytes in residual tumors and alterations in these parameters after neoadjuvant chemotherapy in triple-negative breast cancer: a retrospective multicenter study. Breast cancer research : BCR. 2015;17:124.

72. Balko JM, Nixon M, Gonzalez-Ericsson PI, Pilkinton MA, McDonnell WJ, Sanchez V, et al. Abstract P3-08-15: Immunologic correlates of long-term outcome in the residual disease of triple-negative breast cancer after neoadjuvant chemotherapy. AACR; 2020.

73. Criscitiello C, Bayar MA, Curigliano G, Symmans FW, Desmedt C, Bonnefoi H, et al. A gene signature to predict high tumor-infiltrating lymphocytes after neoadjuvant chemotherapy and outcome in patients with triple-negative breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2018;29(1):162-9.

74. Luen SL, Salgado R, Loi S. Residual disease and immune infiltration as a new surrogate endpoint for TNBC post neoadjuvant chemotherapy. Oncotarget. 2019;10(45):4612-4.

75. Masuda N, Lee SJ, Ohtani S, Im YH, Lee ES, Yokota I, et al. Adjuvant Capecitabine for Breast Cancer after Preoperative Chemotherapy. The New England journal of medicine. 2017;376(22):2147-59.

76. Hamy AS, Pierga JY, Sabaila A, Laas E, Bonsang-Kitzis H, Laurent C, et al. Stromal lymphocyte infiltration after neoadjuvant chemotherapy is associated with aggressive residual disease and lower disease-free survival in HER2-positive breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2017;28(9):2233-40.

77. Force J, Howie LJ, Abbott SE, Bentley R, Marcom PK, Kimmick G, et al. Early Stage HER2-Positive Breast Cancers Not Achieving a pCR From Neoadjuvant

Trastuzumab- or Pertuzumab-Based Regimens Have an Immunosuppressive Phenotype. Clinical breast cancer. 2018;18(5):410-7.

78. Symmans WF, Peintinger F, Hatzis C, Rajan R, Kuerer H, Valero V, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007;25(28):4414-22.

79. Asano Y, Kashiwagi S, Goto W, Takada K, Takahashi K, Hatano T, et al. Prediction of survival after neoadjuvant chemotherapy for breast cancer by evaluation of tumor-infiltrating lymphocytes and residual cancer burden. BMC cancer. 2017;17(1):888.

80. Egelston CA, Avalos C, Tu TY, Simons DL, Jimenez G, Jung JY, et al. Human breast tumor-infiltrating CD8(+) T cells retain polyfunctionality despite PD-1 expression. Nature communications. 2018;9(1):4297.

81. Burugu S, Asleh-Aburaya K, Nielsen TO. Immune infiltrates in the breast cancer microenvironment: detection, characterization and clinical implication. Breast cancer (Tokyo, Japan). 2017;24(1):3-15.

82. Mohammed ZM, Going JJ, Edwards J, McMillan DC. The role of the tumour inflammatory cell infiltrate in predicting recurrence and survival in patients with primary operable breast cancer. Cancer treatment reviews. 2012;38(8):943-55.

83. Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. Annals of oncology : official journal of the European Society for Medical Oncology. 2014;25(8):1536-43.

84. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(15):1949-55.

85. Bohling SD, Allison KH. Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2008;21(12):1527-32.

86. Shou J, Zhang Z, Lai Y, Chen Z, Huang J. Worse outcome in breast cancer with higher tumor-infiltrating FOXP3+ Tregs: a systematic review and meta-analysis. BMC cancer. 2016;16(1):687.

87. West NR, Kost SE, Martin SD, Milne K, Deleeuw RJ, Nelson BH, et al. Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. British journal of cancer. 2013;108(1):155-62.

88. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annual review of immunology. 2010;28:445-89.

89. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. The Journal of clinical investigation. 2013;123(7):2873-92.

90. Kaewkangsadan V, Verma C, Eremin JM, Cowley G, Ilyas M, Eremin O. Crucial Contributions by T Lymphocytes (Effector, Regulatory, and Checkpoint Inhibitor) and Cytokines (TH1, TH2, and TH17) to a Pathological Complete Response Induced by

Neoadjuvant Chemotherapy in Women with Breast Cancer. Journal of immunology research. 2016;2016:4757405.

91. Gonda K, Shibata M, Ohtake T, Matsumoto Y, Tachibana K, Abe N, et al. Myeloid-derived suppressor cells are increased and correlated with type 2 immune responses, malnutrition, inflammation, and poor prognosis in patients with breast cancer. Oncology letters. 2017;14(2):1766-74.

92. Wesolowski R, Duggan MC, Stiff A, Markowitz J, Trikha P, Levine KM, et al. Circulating myeloid-derived suppressor cells increase in patients undergoing neo-adjuvant chemotherapy for breast cancer. Cancer immunology, immunotherapy : CII. 2017;66(11):1437-47.

93. Markowitz J, Wesolowski R, Papenfuss T, Brooks TR, Carson WE, 3rd. Myeloid-derived suppressor cells in breast cancer. Breast cancer research and treatment. 2013;140(1):13-21.

94. Garaud S, Buisseret L, Solinas C, Gu-Trantien C, de Wind A, Van den Eynden G, et al. Tumor infiltrating B-cells signal functional humoral immune responses in breast cancer. JCI insight. 2019;5.

95. Buisseret L, Garaud S, de Wind A, Van den Eynden G, Boisson A, Solinas C, et al. Tumor-infiltrating lymphocyte composition, organization and PD-1/ PD-L1 expression are linked in breast cancer. Oncoimmunology. 2017;6(1):e1257452.

96. Mahmoud SM, Lee AH, Paish EC, Macmillan RD, Ellis IO, Green AR. The prognostic significance of B lymphocytes in invasive carcinoma of the breast. Breast cancer research and treatment. 2012;132(2):545-53.

97. Largeot A, Pagano G, Gonder S, Moussay E, Paggetti J. The B-side of Cancer Immunity: The Underrated Tune. Cells. 2019;8(5).

98. Ishigami E, Sakakibara M, Sakakibara J, Masuda T, Fujimoto H, Hayama S, et al. Coexistence of regulatory B cells and regulatory T cells in tumor-infiltrating lymphocyte aggregates is a prognostic factor in patients with breast cancer. Breast cancer (Tokyo, Japan). 2019;26(2):180-9.

99. Williams CB, Yeh ES, Soloff AC. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. NPJ breast cancer. 2016;2.

100. Zhao X, Qu J, Sun Y, Wang J, Liu X, Wang F, et al. Prognostic significance of tumor-associated macrophages in breast cancer: a meta-analysis of the literature. Oncotarget. 2017;8(18):30576-86.

101. Dieci MV, Radosevic-Robin N, Fineberg S, van den Eynden G, Ternes N, Penault-Llorca F, et al. Update on tumor-infiltrating lymphocytes (TILs) in breast cancer, including recommendations to assess TILs in residual disease after neoadjuvant therapy and in carcinoma in situ: A report of the International Immuno-Oncology Biomarker Working Group on Breast Cancer. Seminars in cancer biology. 2018;52(Pt 2):16-25.

102. Acs B, Rimm DL. Not Just Digital Pathology, Intelligent Digital Pathology. JAMA oncology. 2018;4(3):403-4.

103. Bera K, Schalper KA, Rimm DL, Velcheti V, Madabhushi A. Artificial intelligence in digital pathology - new tools for diagnosis and precision oncology. Nature reviews Clinical oncology. 2019;16(11):703-15.

104. Klauschen F, Muller KR, Binder A, Bockmayr M, Hagele M, Seegerer P, et al. Scoring of tumor-infiltrating lymphocytes: From visual estimation to machine learning. Seminars in cancer biology. 2018;52(Pt 2):151-7.

105. Bai Y, Acs B, Zugazagoitia J, Martinez-Morilla S, Ahmed FS, Rimm DL. Abstract P3-08-12: An open source, automated tumor infiltrating lymphocyte algorithm for prognosis in triple-negative breast cancer. AACR; 2020.

106. Acs B, Ahmed FS, Gupta S, Wong PF, Gartrell RD, Sarin Pradhan J, et al. An open source automated tumor infiltrating lymphocyte algorithm for prognosis in melanoma. Nature communications. 2019;10(1):5440.

107. Keren L, Bosse M, Marquez D, Angoshtari R, Jain S, Varma S, et al. A Structured Tumor-Immune Microenvironment in Triple Negative Breast Cancer Revealed by Multiplexed Ion Beam Imaging. Cell. 2018;174(6):1373-87.e19.

108. Mezheyeuski A, Bergsland CH, Backman M, Djureinovic D, Sjoblom T, Bruun J, et al. Multispectral imaging for quantitative and compartment-specific immune infiltrates reveals distinct immune profiles that classify lung cancer patients. The Journal of pathology. 2018;244(4):421-31.

109. Hackl H, Charoentong P, Finotello F, Trajanoski Z. Computational genomics tools for dissecting tumour-immune cell interactions. Nature reviews Genetics. 2016;17(8):441-58.

110. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. Nature methods. 2015;12(5):453-7.

111. Yoshihara K, Shahmoradgoli M, Martinez E, Vegesna R, Kim H, Torres-Garcia W, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. Nature communications. 2013;4:2612.

112. Becht E, Giraldo NA, Lacroix L, Buttard B, Elarouci N, Petitprez F, et al. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. Genome biology. 2016;17(1):218.

113. Li B, Severson E, Pignon JC, Zhao H, Li T, Novak J, et al. Comprehensive analyses of tumor immunity: implications for cancer immunotherapy. Genome biology. 2016;17(1):174.

114. Aran D, Hu Z, Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. Genome biology. 2017;18(1):220.

115. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(43):15545-50.

116. Azizi E, Carr AJ, Plitas G, Cornish AE, Konopacki C, Prabhakaran S, et al. Single-Cell Map of Diverse Immune Phenotypes in the Breast Tumor Microenvironment. Cell. 2018;174(5):1293-308.e36.

117. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. Nature reviews Immunology. 2018;18(1):35-45.

118. Yofe I, Dahan R, Amit I. Single-cell genomic approaches for developing the next generation of immunotherapies. Nature medicine. 2020;26(2):171-7.

119. Iglesia MD, Parker JS, Hoadley KA, Serody JS, Perou CM, Vincent BG. Genomic Analysis of Immune Cell Infiltrates Across 11 Tumor Types. Journal of the National Cancer Institute. 2016;108(11).

120. Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang TH, et al. The Immune Landscape of Cancer. Immunity. 2018;48(4):812-30.e14.

121. Sota Y, Naoi Y, Tsunashima R, Kagara N, Shimazu K, Maruyama N, et al. Construction of novel immune-related signature for prediction of pathological complete response to neoadjuvant chemotherapy in human breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2014;25(1):100-6.

122. Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012;30(16):1996-2004.

123. Fasching PA, Denkert C, Benz S, Weber KE, Szeto C, Budczies J, et al. Abstract PD5-08: Tumor immune-cell activity assessed by RNAseq is an independent predictor of therapy response and prognosis after neoadjuvant chemotherapy in HER2 negative breast cancer patients-An analysis of the GeparSepto trial. AACR; 2020.

124. Fumagalli D, Venet D, Ignatiadis M, Azim HA, Jr., Maetens M, Rothe F, et al. RNA Sequencing to Predict Response to Neoadjuvant Anti-HER2 Therapy: A Secondary Analysis of the NeoALTTO Randomized Clinical Trial. JAMA oncology. 2017;3(2):227-34.

125. Perez EA, Thompson EA, Ballman KV, Anderson SK, Asmann YW, Kalari KR, et al. Genomic analysis reveals that immune function genes are strongly linked to clinical outcome in the North Central Cancer Treatment Group n9831 Adjuvant Trastuzumab Trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(7):701-8.

126. Kim S-R, Gavin PG, Pogue-Geile KL, Song N, Finnigan M, Bandos H, et al. A surrogate gene expression signature of tumor infiltrating lymphocytes (TILs) predicts degree of benefit from trastuzumab added to standard adjuvant chemotherapy in NSABP (NRG) trial B-31 for HER2+ breast cancer. AACR; 2015.

127. Nederlof I, De Bortoli D, Bareche Y, Nguyen B, de Maaker M, Hooijer GKJ, et al. Comprehensive evaluation of methods to assess overall and cell-specific immune infiltrates in breast cancer. Breast cancer research : BCR. 2019;21(1):151.

128. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annual review of immunology. 2008;26:677-704.

129. Inaguma S, Wang Z, Lasota J, Sarlomo-Rikala M, McCue PA, Ikeda H, et al. Comprehensive Immunohistochemical Study of Programmed Cell Death Ligand 1 (PD-L1): Analysis in 5536 Cases Revealed Consistent Expression in Trophoblastic Tumors. The American journal of surgical pathology. 2016;40(8):1133-42.

130. Simon S, Labarriere N. PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? Oncoimmunology. 2017;7(1):e1364828.

131. Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. The Journal of clinical investigation. 2014;124(5):2246-59.

132. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. Journal of immunology (Baltimore, Md : 1950). 2002;169(10):5538-45.

133. Ma W, Gilligan BM, Yuan J, Li T. Current status and perspectives in translational biomarker research for PD-1/PD-L1 immune checkpoint blockade therapy. Journal of hematology & oncology. 2016;9(1):47.

134. Qin W, Hu L, Zhang X, Jiang S, Li J, Zhang Z, et al. The Diverse Function of PD-1/PD-L Pathway Beyond Cancer. Frontiers in immunology. 2019;10:2298.

135. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nature reviews Immunology. 2004;4(5):336-47.

136. Wei SC, Duffy CR, Allison JP. Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. Cancer discovery. 2018;8(9):1069-86.

137. Vranic S, Cyprian FS, Gatalica Z, Palazzo J. PD-L1 status in breast cancer: Current view and perspectives. Seminars in cancer biology. 2019.

138. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. Cell. 2015;161(2):205-14.

139. Sharma P, Allison JP. The future of immune checkpoint therapy. Science (New York, NY). 2015;348(6230):56-61.

140. Zerdes I, Matikas A, Bergh J, Rassidakis GZ, Foukakis T. Genetic, transcriptional and post-translational regulation of the programmed death protein ligand 1 in cancer: biology and clinical correlations. Oncogene. 2018;37(34):4639-61.

141. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. The New England journal of medicine. 2015;372(4):311-9.

142. Goodman AM, Piccioni D, Kato S, Boichard A, Wang HY, Frampton G, et al. Prevalence of PDL1 Amplification and Preliminary Response to Immune Checkpoint Blockade in Solid Tumors. JAMA oncology. 2018;4(9):1237-44.

143. Bastaki S, Irandoust M, Ahmadi A, Hojjat-Farsangi M, Ambrose P, Hallaj S, et al. PD-L1/PD-1 axis as a potent therapeutic target in breast cancer. Life sciences. 2020;247:117437.

144. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell. 1999;98(3):295-303.

145. Rebe C, Ghiringhelli F. STAT3, a Master Regulator of Anti-Tumor Immune Response. Cancers. 2019;11(9).

146. Kinter AL, Godbout EJ, McNally JP, Sereti I, Roby GA, O'Shea MA, et al. The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. Journal of immunology (Baltimore, Md : 1950). 2008;181(10):6738-46.

147. Oestreich KJ, Yoon H, Ahmed R, Boss JM. NFATc1 regulates PD-1 expression upon T cell activation. Journal of immunology (Baltimore, Md : 1950). 2008;181(7):4832-9.

148. Stovgaard ES, Dyhl-Polk A, Roslind A, Balslev E, Nielsen D. PD-L1 expression in breast cancer: expression in subtypes and prognostic significance: a systematic review. Breast cancer research and treatment. 2019;174(3):571-84.

149. Zhang M, Sun H, Zhao S, Wang Y, Pu H, Wang Y, et al. Expression of PD-L1 and prognosis in breast cancer: a meta-analysis. Oncotarget. 2017;8(19):31347-54.

150. Kim HM, Lee J, Koo JS. Clinicopathological and prognostic significance of programmed death ligand-1 expression in breast cancer: a meta-analysis. BMC cancer. 2017;17(1):690.

151. Bae SB, Cho HD, Oh MH, Lee JH, Jang SH, Hong SA, et al. Expression of Programmed Death Receptor Ligand 1 with High Tumor-Infiltrating Lymphocytes Is Associated with Better Prognosis in Breast Cancer. Journal of breast cancer. 2016;19(3):242-51.

152. Beckers RK, Selinger CI, Vilain R, Madore J, Wilmott JS, Harvey K, et al. Programmed death ligand 1 expression in triple-negative breast cancer is associated with tumour-infiltrating lymphocytes and improved outcome. Histopathology. 2016;69(1):25-34.

153. Huang W, Ran R, Shao B, Li H. Prognostic and clinicopathological value of PD-L1 expression in primary breast cancer: a meta-analysis. Breast cancer research and treatment. 2019;178(1):17-33.

154. Bertucci F, Finetti P, Colpaert C, Mamessier E, Parizel M, Dirix L, et al. PDL1 expression in inflammatory breast cancer is frequent and predicts for the pathological response to chemotherapy. Oncotarget. 2015;6(15):13506-19.

155. Wang ZQ, Milne K, Derocher H, Webb JR, Nelson BH, Watson PH. PD-L1 and intratumoral immune response in breast cancer. Oncotarget. 2017;8(31):51641-51.

156. Schalper KA, Velcheti V, Carvajal D, Wimberly H, Brown J, Pusztai L, et al. In situ tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014;20(10):2773-82.

157. Teng MW, Ngiow SF, Ribas A, Smyth MJ. Classifying Cancers Based on T-cell Infiltration and PD-L1. Cancer research. 2015;75(11):2139-45.

158. Ribas A. Adaptive Immune Resistance: How Cancer Protects from Immune Attack. Cancer discovery. 2015;5(9):915-9.

159. Hegde PS, Chen DS. Top 10 Challenges in Cancer Immunotherapy. Immunity. 2020;52(1):17-35.

160. Matikas A, Zerdes I, Lovrot J, Richard F, Sotiriou C, Bergh J, et al. Prognostic Implications of PD-L1 Expression in Breast Cancer: Systematic Review and Meta-analysis of Immunohistochemistry and Pooled Analysis of Transcriptomic Data. Clinical cancer research : an official journal of the American Association for Cancer Research. 2019;25(18):5717-26.

161. Sabatier R, Finetti P, Mamessier E, Adelaide J, Chaffanet M, Ali HR, et al. Prognostic and predictive value of PDL1 expression in breast cancer. Oncotarget. 2015;6(7):5449-64.

162. Matikas A, Lovrot J, Ramberg A, Eriksson M, Lindsten T, Lekberg T, et al. Dynamic evaluation of the immune infiltrate and immune function genes as predictive markers for neoadjuvant chemotherapy in hormone receptor positive, HER2 negative breast cancer. Oncoimmunology. 2018;7(9):e1466017.

163. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. The New England journal of medicine. 2018;379(22):2108-21.

164. Cortés J, Lipatov O, Im S-A, Gonçalves A, Lee K, Schmid P, et al. KEYNOTE-119: Phase III study of pembrolizumab (pembro) versus single-agent chemotherapy (chemo) for metastatic triple negative breast cancer (mTNBC). Annals of Oncology. 2019;30:v859-v60.

165. Du Q, Che J, Jiang X, Li L, Luo X, Li Q. PD-L1 Acts as a Promising Immune Marker to Predict the Response to Neoadjuvant Chemotherapy in Breast Cancer Patients. Clinical breast cancer. 2020;20(1):e99-e111.

166. Pelekanou V, Barlow WE, Nahleh ZA, Wasserman B, Lo YC, von Wahlde MK, et al. Tumor-Infiltrating Lymphocytes and PD-L1 Expression in Pre- and Posttreatment Breast Cancers in the SWOG S0800 Phase II Neoadjuvant Chemotherapy Trial. Molecular cancer therapeutics. 2018;17(6):1324-31.

167. Harbeck N, Nitz U, Matthias C, Kates R, Braun M, Kümmel S, et al. Abstract P1-09-05: The role of immune and apoptosis markers for prediction of pCR in the WSG-ADAPT HER2+/HR+ phase II trial evaluating 12-weeks of neoadjuvant TDM1±endocrine therapy (ET) versus T+ ET in HER2-positive hormone-receptor-positive early breast cancer (EBC). AACR; 2017.

168. Chumsri S, Hinerfeld D, Kachergus JM, Ma Y, Brauer HA, Warren S, et al. Abstract PD5-06: Digital spatial mapping of the immune landscape of triple negative breast cancer reveals novel features of immune-tumor cell interaction. AACR; 2020.

169. Sanchez K, Kim I, Chang S, Martel M, Wu Y, Bernard B, et al. Abstract P6-10-09: Multispectral immunofluorescence (mIF) to detect dynamic changes in PD-L1 expression, immune cell (IC) infiltration, and tumor-IC interactions in primary breast cancer (BC) immuno-oncology (IO) clinical trials. AACR; 2020.

170. Hirsch FR, McElhinny A, Stanforth D, Ranger-Moore J, Jansson M, Kulangara K, et al. PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer. 2017;12(2):208-22.

171. Tsao MS, Kerr KM, Kockx M, Beasley MB, Borczuk AC, Botling J, et al. PD-L1 Immunohistochemistry Comparability Study in Real-Life Clinical Samples: Results of Blueprint Phase 2 Project. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer. 2018;13(9):1302-11.

172. Martinez-Morilla S, McGuire J, Gaule P, Moore L, Acs B, Cougot D, et al. Quantitative assessment of PD-L1 as an analyte in immunohistochemistry diagnostic assays using a standardized cell line tissue microarray. Laboratory investigation; a journal of technical methods and pathology. 2020;100(1):4-15.

173. Torlakovic E, Lim HJ, Adam J, Barnes P, Bigras G, Chan AWH, et al. "Interchangeability" of PD-L1 immunohistochemistry assays: a meta-analysis of diagnostic accuracy. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2020;33(1):4-17.

174. Rimm DL, Han G, Taube JM, Yi ES, Bridge JA, Flieder DB, et al. A Prospective, Multi-institutional, Pathologist-Based Assessment of 4 Immunohistochemistry

Assays for PD-L1 Expression in Non-Small Cell Lung Cancer. JAMA oncology. 2017;3(8):1051-8.

175. Sun WY, Lee YK, Koo JS. Expression of PD-L1 in triple-negative breast cancer based on different immunohistochemical antibodies. Journal of translational medicine. 2016;14(1):173.

176. Miglietta F, Griguolo G, Guarneri V, Dieci MV. Programmed Cell Death Ligand 1 in Breast Cancer: Technical Aspects, Prognostic Implications, and Predictive Value. The oncologist. 2019;24(11):e1055-e69.

177. Karnik T, Kimler BF, Fan F, Tawfik O. PD-L1 in breast cancer: comparative analysis of 3 different antibodies. Human pathology. 2018;72:28-34.

178. Noske A, Ammann J, Wagner D-C, Denkert C, Lebeau A, Sinn P, et al. Reproducibility and concordance of 4 clinically developed programmed death-ligand 1 (PD-L1) immunohistochemistry (IHC) assays in triple negative breast cancer (TNBC). Annals of Oncology. 2019;30:v130-v1.

179. Scott M, Scorer P, Barker C, Al-Masri H. 10O Comparison of patient populations identified by different PD-L1 assays in in triple-negative breast cancer (TNBC). Annals of Oncology. 2019;30(Supplement_3):mdz095. 09.

180. Carter JM, Polley M-YC, Sinnwell JP, Leon-Ferre RA, Couch FJ, Kalari KR, et al. Abstract PD1-08: Frequency, characteristics and prognostic factors of PD-L1+ triple negative breast cancer using the PD-L1 SP142 companion assay. AACR; 2020.

181. Rugo H, Loi S, Adams S, Schmid P, Schneeweiss A, Barrios CH, et al. Abstract PD1-07: Exploratory analytical harmonization of PD-L1 immunohistochemistry assays in advanced triple-negative breast cancer: A retrospective substudy of IMpassion130. AACR; 2020.

182. Kim HN, Jang J, Heo YJ, Kim B, Jung H, Jang Y, et al. PD-L1 expression in gastric cancer determined by digital image analyses: pitfalls and correlation with pathologist interpretation. Virchows Archiv : an international journal of pathology. 2020;476(2):243-50.

183. Silva MA, Triltsch N, Leis S, Kanchev I, Tan TH, Van Peel B, et al. Biomarker recommendation for PD-1/PD-L1 immunotherapy development in pediatric cancer based on digital image analysis of PD-L1 and immune cells. The journal of pathology Clinical research. 2020.

184. Muenst S, Soysal SD, Gao F, Obermann EC, Oertli D, Gillanders WE. The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. Breast cancer research and treatment. 2013;139(3):667-76.

185. Sun S, Fei X, Mao Y, Wang X, Garfield DH, Huang O, et al. PD-1(+) immune cell infiltration inversely correlates with survival of operable breast cancer patients. Cancer immunology, immunotherapy : CII. 2014;63(4):395-406.

186. Noske A, Mobus V, Weber K, Schmatloch S, Weichert W, Kohne CH, et al. Relevance of tumour-infiltrating lymphocytes, PD-1 and PD-L1 in patients with high-risk, nodal-metastasised breast cancer of the German Adjuvant Intergroup Node-positive study. European journal of cancer (Oxford, England : 1990). 2019;114:76-88.

187. Yeong J, Lim JCT, Lee B, Li H, Ong CCH, Thike AA, et al. Prognostic value of CD8 + PD-1+ immune infiltrates and PDCD1 gene expression in triple negative breast cancer. Journal for immunotherapy of cancer. 2019;7(1):34.

188. Brockhoff G, Seitz S, Weber F, Zeman F, Klinkhammer-Schalke M, Ortmann O, et al. The presence of PD-1 positive tumor infiltrating lymphocytes in triple negative breast cancers is associated with a favorable outcome of disease. Oncotarget. 2018;9(5):6201-12.

189. Dill EA, Gru AA, Atkins KA, Friedman LA, Moore ME, Bullock TN, et al. PD-L1 Expression and Intratumoral Heterogeneity Across Breast Cancer Subtypes and Stages: An Assessment of 245 Primary and 40 Metastatic Tumors. The American journal of surgical pathology. 2017;41(3):334-42.

190. Tawfik O, Kimler BF, Karnik T, Shehata P. Clinicopathological correlation of PD-L1 expression in primary and metastatic breast cancer and infiltrating immune cells. Human pathology. 2018;80:170-8.

191. Manson QF, Schrijver W, Ter Hoeve ND, Moelans CB, van Diest PJ. Frequent discordance in PD-1 and PD-L1 expression between primary breast tumors and their matched distant metastases. Clinical & experimental metastasis. 2019;36(1):29-37.

192. Li M, Li A, Zhou S, Xu Y, Xiao Y, Bi R, et al. Heterogeneity of PD-L1 expression in primary tumors and paired lymph node metastases of triple negative breast cancer. BMC cancer. 2018;18(1):4.

193. Rugo HS, Loi S, Adams S, Schmid P, Schneeweiss A, Barrios CH, et al. Performance of PD-L1 immunohistochemistry (IHC) assays in unresectable locally advanced or metastatic triple-negative breast cancer (mTNBC): post-hoc analysis of Impassion130. Annals of Oncology. 2019;30:v858-v9.

194. Cerbelli B, Pernazza A, Botticelli A, Fortunato L, Monti M, Sciattella P, et al. PD-L1 Expression in TNBC: A Predictive Biomarker of Response to Neoadjuvant Chemotherapy? BioMed research international. 2017;2017:1750925.

195. McLemore LE, Janakiram M, Albanese J, Shapiro N, Lo Y, Zang X, et al. An Immunoscore Using PD-L1, CD68, and Tumor-infiltrating Lymphocytes (TILs) to Predict Response to Neoadjuvant Chemotherapy in Invasive Breast Cancer. Applied immunohistochemistry & molecular morphology : AIMM. 2018;26(9):611-9.

196. Chen S, Wang RX, Liu Y, Yang WT, Shao ZM. PD-L1 expression of the residual tumor serves as a prognostic marker in local advanced breast cancer after neoadjuvant chemotherapy. International journal of cancer. 2017;140(6):1384-95.

197. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. Cell death and differentiation. 2014;21(1):15-25.

198. Vanmeerbeek I, Sprooten J, De Ruysscher D, Tejpar S, Vandenberghe P, Fucikova J, et al. Trial watch: chemotherapy-induced immunogenic cell death in immuno-oncology. Oncoimmunology. 2020;9(1):1703449.

199. La-Beck NM, Jean GW, Huynh C, Alzghari SK, Lowe DB. Immune Checkpoint Inhibitors: New Insights and Current Place in Cancer Therapy. Pharmacotherapy. 2015;35(10):963-76.

200. Duan J, Cui L, Zhao X, Bai H, Cai S, Wang G, et al. Use of Immunotherapy With Programmed Cell Death 1 vs Programmed Cell Death Ligand 1 Inhibitors in Patients With Cancer: A Systematic Review and Meta-analysis. JAMA oncology. 2019.

201. Adams S, Gatti-Mays ME, Kalinsky K, Korde LA, Sharon E, Amiri-Kordestani L, et al. Current Landscape of Immunotherapy in Breast Cancer: A Review. JAMA oncology. 2019.

202. Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. The Lancet Oncology. 2020;21(1):44-59.

203. Narayan P, Wahby S, Gao JJ, Amiri-Kordestani L, Ibrahim A, Bloomquist E, et al. FDA Approval Summary: Atezolizumab plus paclitaxel protein-bound for the treatment of patients with advanced or metastatic TNBC whose tumors express PD-L1. Clinical cancer research : an official journal of the American Association for Cancer Research. 2020.

204. Adams S, Loi S, Toppmeyer D, Cescon DW, De Laurentiis M, Nanda R, et al. Pembrolizumab monotherapy for previously untreated, PD-L1-positive, metastatic triple-negative breast cancer: cohort B of the phase II KEYNOTE-086 study. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(3):405-11.

205. Adams S, Schmid P, Rugo HS, Winer EP, Loirat D, Awada A, et al. Pembrolizumab monotherapy for previously treated metastatic triple-negative breast cancer: cohort A of the phase II KEYNOTE-086 study. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(3):397-404.

206. Voorwerk L, Slagter M, Horlings HM, Sikorska K, van de Vijver KK, de Maaker M, et al. Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial. Nature medicine. 2019;25(6):920-8.

207. Schmid P, Cortes J, Pusztai L, McArthur H, Kümmel S, Bergh J, et al. Pembrolizumab for Early Triple-Negative Breast Cancer. New England Journal of Medicine. 2020;382(9):810-21.

208. Schmid P, Salgado R, Park Y, Muñoz-Couselo E, Kim S, Sohn J, et al. Pembrolizumab plus chemotherapy as neoadjuvant treatment for high-risk, early-stage triple-negative breast cancer: results from the phase 1b open-label, multicohort KEYNOTE-173 study. Annals of Oncology. 2020.

209. Nanda R, Liu MC, Yau C, Shatsky R, Pusztai L, Wallace A, et al. Effect of Pembrolizumab Plus Neoadjuvant Chemotherapy on Pathologic Complete Response in Women With Early-Stage Breast Cancer: An Analysis of the Ongoing Phase 2 Adaptively Randomized I-SPY2 Trial. JAMA oncology. 2020.

210. Loibl S, Untch M, Burchardi N, Huober J, Sinn BV, Blohmer JU, et al. A randomised phase II study investigating durvalumab in addition to an anthracycline taxane-based neoadjuvant therapy in early triple-negative breast cancer: clinical results and biomarker analysis of GeparNuevo study. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(8):1279-88.

211. Gianni L, Huang C, Egle D, Bermejo B, editors. Pathologic complete response (pCR) to neoadjuvant treatment with or without atezolizumab in triple negative, early high-risk and locally advanced breast cancer. NeoTRIPaPDL1 Michelangelo randomized study. San Antonio Breast Cancer Symposium; 2019.

212. Loi S, Giobbie-Hurder A, Gombos A, Bachelot T, Hui R, Curigliano G, et al. Pembrolizumab plus trastuzumab in trastuzumab-resistant, advanced, HER2-positive breast

cancer (PANACEA): a single-arm, multicentre, phase 1b-2 trial. The Lancet Oncology. 2019;20(3):371-82.

213. Emens L, Esteva F, Beresford M, Saura C, De Laurentiis M, Kim S, et al. 305O Overall survival (OS) in KATE2, a phase II study of programmed death ligand 1 (PD-L1) inhibitor atezolizumab (atezo)+ trastuzumab emtansine (T-DM1) vs placebo (pbo)+ T-DM1 in previously treated HER2+ advanced breast cancer (BC). Annals of Oncology. 2019;30(Supplement_5):mdz242.

214. Griguolo G, Pascual T, Dieci MV, Guarneri V, Prat A. Interaction of host immunity with HER2-targeted treatment and tumor heterogeneity in HER2-positive breast cancer. Journal for immunotherapy of cancer. 2019;7(1):90.

215. Esteva FJ, Hubbard-Lucey VM, Tang J, Pusztai L. Immunotherapy and targeted therapy combinations in metastatic breast cancer. The Lancet Oncology. 2019;20(3):e175-e86.

216. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. Nature reviews Cancer. 2019;19(3):133-50.

217. Shen X, Zhao B. Efficacy of PD-1 or PD-L1 inhibitors and PD-L1 expression status in cancer: meta-analysis. BMJ (Clinical research ed). 2018;362:k3529.

218. Gonzalez-Ericsson PI, Stovgaard ES, Sua LF, Reisenbichler E, Kos Z, Carter JM, et al. The path to a better biomarker: application of a risk management framework for the implementation of PD-L1 and TILs as immuno-oncology biomarkers into breast cancer clinical trials and daily practice. The Journal of pathology. 2020.

219. Emens L, Loi S, Rugo H, Schneeweiss A, Diéras V, Iwata H, et al. Abstract GS1-04: IMpassion130: Efficacy in immune biomarker subgroups from the global, randomized, double-blind, placebo-controlled, phase III study of atezolizumab+ nab-paclitaxel in patients with treatment-naïve, locally advanced or metastatic triple-negative breast cancer. AACR; 2019.

220. Emens LA, Cruz C, Eder JP, Braiteh F, Chung C, Tolaney SM, et al. Long-term Clinical Outcomes and Biomarker Analyses of Atezolizumab Therapy for Patients With Metastatic Triple-Negative Breast Cancer: A Phase 1 Study. JAMA oncology. 2019;5(1):74-82.

221. McLaughlin J, Han G, Schalper KA, Carvajal-Hausdorf D, Pelekanou V, Rehman J, et al. Quantitative Assessment of the Heterogeneity of PD-L1 Expression in Non-Small-Cell Lung Cancer. JAMA oncology. 2016;2(1):46-54.

222. Loi S, Winer E, Lipatov O, Im S-A, Goncalves A, Cortes J, et al. Abstract PD5-03: Relationship between tumor-infiltrating lymphocytes (TILs) and outcomes in the KEYNOTE-119 study of pembrolizumab vs chemotherapy for previously treated metastatic triple-negative breast cancer (mTNBC). AACR; 2020.

223. Prat A, Navarro A, Pare L, Reguart N, Galvan P, Pascual T, et al. Immune-Related Gene Expression Profiling After PD-1 Blockade in Non-Small Cell Lung Carcinoma, Head and Neck Squamous Cell Carcinoma, and Melanoma. Cancer research. 2017;77(13):3540-50.

224. Pare L, Pascual T, Segui E, Teixido C, Gonzalez-Cao M, Galvan P, et al. Association between PD1 mRNA and response to anti-PD1 monotherapy across multiple cancer types. Annals of oncology : official journal of the European Society for Medical Oncology. 2018;29(10):2121-8.

225. Lee JS, Ruppin E. Multiomics Prediction of Response Rates to Therapies to Inhibit Programmed Cell Death 1 and Programmed Cell Death 1 Ligand 1. JAMA oncology. 2019.

226. Loibl S, Sinn B, Karn T, Untch M, Treue D, Sinn H, et al. Abstract PD2-07: mRNA signatures predict response to durvalumab therapy in triple negative breast cancer (TNBC)–Results of the translational biomarker programme of the neoadjuvant double-blind placebo controlled GeparNuevo trial. AACR; 2019.

227. Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton GM, Miller V, et al. Tumor Mutational Burden as an Independent Predictor of Response to Immunotherapy in Diverse Cancers. Molecular cancer therapeutics. 2017;16(11):2598-608.

228. Hellmann MD, Ciuleanu TE, Pluzanski A, Lee JS, Otterson GA, Audigier-Valette C, et al. Nivolumab plus Ipilimumab in Lung Cancer with a High Tumor Mutational Burden. The New England journal of medicine. 2018;378(22):2093-104.

229. Endris V, Buchhalter I, Allgauer M, Rempel E, Lier A, Volckmar AL, et al. Measurement of tumor mutational burden (TMB) in routine molecular diagnostics: in silico and real-life analysis of three larger gene panels. International journal of cancer. 2019;144(9):2303-12.

230. Truesdell J, Miller VA, Fabrizio D. Approach to evaluating tumor mutational burden in routine clinical practice. Translational lung cancer research. 2018;7(6):678-81.

231. Loibl S, Sinn BV, Karn T, Untch M, Sinn H-P, Weber KE, et al. Exome analysis of oncogenic pathways and tumor mutational burden (TMB) in triple-negative breast cancer (TNBC): Results of the translational biomarker program of the neoadjuvant double-blind placebo controlled GeparNuevo trial. American Society of Clinical Oncology; 2019.

232. Lu S, Stein JE, Rimm DL, Wang DW, Bell JM, Johnson DB, et al. Comparison of Biomarker Modalities for Predicting Response to PD-1/PD-L1 Checkpoint Blockade: A Systematic Review and Meta-analysis. JAMA oncology. 2019.

233. Ren D, Hua Y, Yu B, Ye X, He Z, Li C, et al. Predictive biomarkers and mechanisms underlying resistance to PD1/PD-L1 blockade cancer immunotherapy. Molecular cancer. 2020;19(1):19.

234. Lundberg A, Lindstrom LS, Harrell JC, Falato C, Carlson JW, Wright PK, et al. Gene Expression Signatures and Immunohistochemical Subtypes Add Prognostic Value to Each Other in Breast Cancer Cohorts. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017;23(24):7512-20.

235. Altman DG, De Stavola BL, Love SB, Stepniewska KA. Review of survival analyses published in cancer journals. British journal of cancer. 1995;72(2):511-8.

236. Liu J, Lichtenberg T, Hoadley KA, Poisson LM, Lazar AJ, Cherniack AD, et al. An Integrated TCGA Pan-Cancer Clinical Data Resource to Drive High-Quality Survival Outcome Analytics. Cell. 2018;173(2):400-16.e11.

237. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science signaling. 2013;6(269):pl1.

238. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer discovery. 2012;2(5):401-4.

239. Hatschek T, Carlsson L, Einbeigi Z, Lidbrink E, Linderholm B, Lindh B, et al. Individually tailored treatment with epirubicin and paclitaxel with or without capecitabine as first-line chemotherapy in metastatic breast cancer: a randomized multicenter trial. Breast cancer research and treatment. 2012;131(3):939-47.

240. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. Nucleic acids research. 2003;31(4):e15.

241. Bengtsson H, Simpson K, Bullard J, Hansen K. aroma. affymetrix: A generic framework in R for analyzing small to very large Affymetrix data sets in bounded memory. tech report; 2008.

242. Tobin NP, Harrell JC, Lovrot J, Egyhazi Brage S, Frostvik Stolt M, Carlsson L, et al. Molecular subtype and tumor characteristics of breast cancer metastases as assessed by gene expression significantly influence patient post-relapse survival. Annals of oncology : official journal of the European Society for Medical Oncology. 2015;26(1):81-8.

243. Wang K, Singh D, Zeng Z, Coleman SJ, Huang Y, Savich GL, et al. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. Nucleic acids research. 2010;38(18):e178.

244. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC bioinformatics. 2011;12:323.

245. Bullard JH, Purdom E, Hansen KD, Dudoit S. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC bioinformatics. 2010;11:94.

246. Bozovic-Spasojevic I, Zardavas D, Brohee S, Ameye L, Fumagalli D, Ades F, et al. The Prognostic Role of Androgen Receptor in Patients with Early-Stage Breast Cancer: A Meta-analysis of Clinical and Gene Expression Data. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017;23(11):2702-12.

247. Haibe-Kains B, Desmedt C, Loi S, Culhane AC, Bontempi G, Quackenbush J, et al. A three-gene model to robustly identify breast cancer molecular subtypes. Journal of the National Cancer Institute. 2012;104(4):311-25.

248. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009;27(8):1160-7.

249. Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, et al. Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. Cell. 2015;163(2):506-19.

250. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012;486(7403):346-52.

251. Sorlie T, Borgan E, Myhre S, Vollan HK, Russnes H, Zhao X, et al. The importance of gene-centring microarray data. The Lancet Oncology. 2010;11(8):719-20; author reply 20-1.

252. Perou CM, Parker JS, Prat A, Ellis MJ, Bernard PS. Clinical implementation of the intrinsic subtypes of breast cancer. The Lancet Oncology. 2010;11(8):718-9; author reply 20-1.

253. Paquet ER, Hallett MT. Absolute assignment of breast cancer intrinsic molecular subtype. Journal of the National Cancer Institute. 2015;107(1):357.

254. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. The New England journal of medicine. 2004;351(27):2817-26.

255. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415(6871):530-6.

256. Gendoo DM, Ratanasirigulchai N, Schroder MS, Pare L, Parker JS, Prat A, et al. Genefu: an R/Bioconductor package for computation of gene expression-based signatures in breast cancer. Bioinformatics (Oxford, England). 2016;32(7):1097-9.

257. Sonnenblick A, Brohee S, Fumagalli D, Vincent D, Venet D, Ignatiadis M, et al. Constitutive phosphorylated STAT3-associated gene signature is predictive for trastuzumab resistance in primary HER2-positive breast cancer. BMC medicine. 2015;13:177.

258. Huang da W, Sherman BT, Stephens R, Baseler MW, Lane HC, Lempicki RA. DAVID gene ID conversion tool. Bioinformation. 2008;2(10):428-30.

259. Nielsen TO, Parker JS, Leung S, Voduc D, Ebbert M, Vickery T, et al. A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16(21):5222-32.

260. Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G, et al. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(16):5158-65.

261. Loi S, Haibe-Kains B, Majjaj S, Lallemand F, Durbecq V, Larsimont D, et al. PIK3CA mutations associated with gene signature of low mTORC1 signaling and better outcomes in estrogen receptor-positive breast cancer. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(22):10208-13.

262. Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, et al. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(38):13550-5.

263. Chen B, Khodadoust MS, Liu CL, Newman AM, Alizadeh AA. Profiling Tumor Infiltrating Immune Cells with CIBERSORT. Methods in molecular biology (Clifton, NJ). 2018;1711:243-59.

264. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research. 2015;43(7):e47.

265. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61-70.

266. Zerdes I, Wallerius M, Sifakis EG, Wallmann T, Betts S, Bartish M, et al. STAT3 Activity Promotes Programmed-Death Ligand 1 Expression and Suppresses Immune Responses in Breast Cancer. Cancers. 2019;11(10).

267. Straub M, Drecoll E, Pfarr N, Weichert W, Langer R, Hapfelmeier A, et al. CD274/PD-L1 gene amplification and PD-L1 protein expression are common events in squamous cell carcinoma of the oral cavity. Oncotarget. 2016;7(11):12024-34.

268. Balko JM, Schwarz LJ, Luo N, Estrada MV, Giltnane JM, Davila-Gonzalez D, et al. Triple-negative breast cancers with amplification of JAK2 at the 9p24 locus demonstrate JAK2-specific dependence. Science translational medicine. 2016;8(334):334ra53.

269. Roemer MG, Advani RH, Ligon AH, Natkunam Y, Redd RA, Homer H, et al. PD-L1 and PD-L2 Genetic Alterations Define Classical Hodgkin Lymphoma and Predict Outcome. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2016;34(23):2690-7.

270. Follenzi A, Naldini L. HIV-based vectors. Preparation and use. Methods in molecular medicine. 2002;69:259-74.

271. Witt K, Ligtenberg MA, Conti L, Lanzardo S, Ruiu R, Wallmann T, et al. Cripto-1 Plasmid DNA Vaccination Targets Metastasis and Cancer Stem Cells in Murine Mammary Carcinoma. Cancer immunology research. 2018;6(11):1417-25.

272. Casazza A, Fu X, Johansson I, Capparuccia L, Andersson F, Giustacchini A, et al. Systemic and targeted delivery of semaphorin 3A inhibits tumor angiogenesis and progression in mouse tumor models. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(4):741-9.

273. Witt K, Lundqvist A. Evaluation of Breast Cancer and Melanoma Metastasis in Syngeneic Mouse Models. Methods in molecular biology (Clifton, NJ). 2019;1913:197-206.

274. Altman DG, McShane LM, Sauerbrei W, Taube SE. Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK): explanation and elaboration. PLoS medicine. 2012;9(5):e1001216.

275. Green GH, Diggle PJ. On the operational characteristics of the Benjamini and Hochberg False Discovery Rate procedure. Statistical applications in genetics and molecular biology. 2007;6:Article27.

276. Ramasamy A, Mondry A, Holmes CC, Altman DG. Key issues in conducting a meta-analysis of gene expression microarray datasets. PLoS medicine. 2008;5(9):e184.

277. Zerdes I, Sifakis EG, Matikas A, Chretien S, Tobin NP, Hartman J, et al. Programmed death-ligand 1 gene expression is a prognostic marker in early breast cancer and provides additional prognostic value to 21-gene and 70-gene signatures in estrogen receptor-positive disease. Molecular oncology. 2020.

278. Ali HR, Glont SE, Blows FM, Provenzano E, Dawson SJ, Liu B, et al. PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes. Annals of oncology : official journal of the European Society for Medical Oncology. 2015;26(7):1488-93.

279. Ren X, Wu H, Lu J, Zhang Y, Luo Y, Xu Q, et al. PD1 protein expression in tumor infiltrated lymphocytes rather than PDL1 in tumor cells predicts survival in triple-negative breast cancer. Cancer biology & therapy. 2018;19(5):373-80.

280. Heindl A, Sestak I, Naidoo K, Cuzick J, Dowsett M, Yuan Y. Relevance of Spatial Heterogeneity of Immune Infiltration for Predicting Risk of Recurrence After Endocrine Therapy of ER+ Breast Cancer. Journal of the National Cancer Institute. 2018;110(2).

281. Sobral-Leite M, Van de Vijver K, Michaut M, van der Linden R, Hooijer GKJ, Horlings HM, et al. Assessment of PD-L1 expression across breast cancer molecular subtypes, in relation to mutation rate, BRCA1-like status, tumor-infiltrating immune cells and survival. Oncoimmunology. 2018;7(12):e1509820.

282. Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). Proceedings of the National Academy of Sciences of the United States of America. 2008;105(52):20852-7.

283. Sasidharan Nair V, Toor SM, Ali BR, Elkord E. Dual inhibition of STAT1 and STAT3 activation downregulates expression of PD-L1 in human breast cancer cells. Expert opinion on therapeutic targets. 2018;22(6):547-57.

284. Hartley GP, Chow L, Ammons DT, Wheat WH, Dow SW. Programmed Cell Death Ligand 1 (PD-L1) Signaling Regulates Macrophage Proliferation and Activation. Cancer immunology research. 2018.

285. Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer cell. 2011;19(1):31-44.

286. Harada K, Dong X, Estrella JS, Correa AM, Xu Y, Hofstetter WL, et al. Tumor-associated macrophage infiltration is highly associated with PD-L1 expression in gastric adenocarcinoma. Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association. 2018;21(1):31-40.

287. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. Cancer cell. 2013;23(3):277-86.

288. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. Nature reviews Clinical oncology. 2017;14(7):399-416.

289. Qin JJ, Yan L, Zhang J, Zhang WD. STAT3 as a potential therapeutic target in triple negative breast cancer: a systematic review. Journal of experimental & clinical cancer research : CR. 2019;38(1):195.

290. Santoni M, Romagnoli E, Saladino T, Foghini L, Guarino S, Capponi M, et al. Triple negative breast cancer: Key role of Tumor-Associated Macrophages in regulating the activity of anti-PD-1/PD-L1 agents. Biochimica et biophysica acta. 2018;1869(1):78-84.

291. Chretien S, Zerdes I, Bergh J, Matikas A, Foukakis T. Beyond PD-1/PD-L1 Inhibition: What the Future Holds for Breast Cancer Immunotherapy. Cancers. 2019;11(5).

292. Jiang C, Cao S, Li N, Jiang L, Sun T. PD-1 and PD-L1 correlated gene expression profiles and their association with clinical outcomes of breast cancer. Cancer cell international. 2019;19:233.

293. Boussiotis VA. Molecular and Biochemical Aspects of the PD-1 Checkpoint Pathway. The New England journal of medicine. 2016;375(18):1767-78.

294. Anurag M, Zhu M, Huang C, Vasaikar S, Wang J, Hoog J, et al. Immune Checkpoint Profiles in Luminal B Breast Cancer (Alliance). Journal of the National Cancer Institute. 2019.

295. Lindstrom LS, Karlsson E, Wilking UM, Johansson U, Hartman J, Lidbrink EK, et al. Clinically used breast cancer markers such as estrogen receptor, progesterone

receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012;30(21):2601-8.

296. Foukakis T, Astrom G, Lindstrom L, Hatschek T, Bergh J. When to order a biopsy to characterise a metastatic relapse in breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2012;23 Suppl 10:x349-53.